



Cryostorage of the threatened and endemic *Moehringia jankae* Griseb. ex Janka through droplet-vitrification and encapsulation-dehydration

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Abstract

The threatened and European-endemic species *Moehringia jankae* Griseb. ex Janka (Caryophyllaceae) exhibits a critically restricted natural distribution confined solely to the Western Black Sea region. Facing intense adaptive challenges imposed by progressive climate change and increasing anthropogenic disturbances, we assert that cryopreservation constitutes the only feasible and sustainable long-term ex situ conservation strategy for this species. Consequently, the present investigation optimized droplet-vitrification and encapsulation-dehydration methodologies designed to ensure the secure preservation of *M. jankae*, concurrently upholding robust regrowth rates of explants post-liquid nitrogen immersion. Our findings demonstrate maximal regrowth efficiencies: 83% via droplet-vitrification following a 24-hour osmoprotection in 0.5 M sucrose, succeeded by 15 min of PVS2 dehydration; and 85% through encapsulation-dehydration after a 24-hour osmoprotection in 0.75 M sucrose, and 23% moisture content of alginate beads. These biotechnological approaches demonstrate significant utility for the effective ex situ conservation of additional rare or endangered plant species, thereby securing the long-term protection of global biodiversity. To the best of our knowledge, this study represents the first comprehensive documentation of *M. jankae* cryopreservation, thereby establishing critical cryopreservation protocols (droplet vitrification and encapsulation-dehydration) essential for the ex situ conservation of this unique species.

Key message

Optimized droplet-vitrification and encapsulation-dehydration cryopreservation approaches enable effective and sustainable ex situ conservation of the threatened European-endemic *Moehringia jankae*.

Keywords Cryopreservation · Encapsulation-dehydration · Liquid nitrogen · Nodal explants · Vitrification

Introduction

Climate change is impacting biodiversity, with endemic species being especially vulnerable (Manes et al. 2021; Mkala et al. 2023). These species are irreplaceable and require targeted conservation to prevent extinction and

loss of ecosystem functions (Gonçalves-Souza et al. 2020). Their vulnerability stems from climate change, small populations (Staude et al. 2020), habitat destruction (Heinken and Weber 2013), invasive species (Manes et al. 2021), and human activities (Nunez et al. 2019). Without strong mitigation, these threats will worsen (Manes et al. 2021). Countries harboring endemic species hold a global conservation responsibility (Heywood 2019). Research on their ecosystem roles and economic potential is essential for supporting conservation and development (Coelho et al. 2020; Benelli et al. 2024). Since many endemics occur in biodiversity hotspots, focusing efforts there is an effective conservation strategy (Marchese 2015). Consequently, urgent measures including in situ and ex situ programs are needed to protect the world's biodiversity hotspots (Schrader et al. 2024). The long-term ex situ conservation of plant genetic resources aims to preserve the genetic diversity of

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individual accessions while preserving their genetic composition (Engels and Ebert 2021).

Significant advancements have been made in plant cryopreservation, with a primary emphasis on crop genetic resources and other economically valuable species (Kulus and Zalewska 2014; Halmagyi et al. 2017; Kulus 2019; Ruta et al. 2020). Nevertheless, fewer studies are available for rare or endemic plants, which are equally crucial for conservation efforts. Worldwide the most commonly used methods for cryopreservation of various endemic species include droplet-vitrification (Halmagyi et al. 2020), encapsulation-vitrification (Martín et al. 2018), and encapsulation-dehydration (Coelho et al. 2014a; Kulus et al. 2018, 2025). Vitrification is the physical process in which a highly concentrated cryoprotective solution is rapidly cooled to extremely low temperatures, solidifying into a metastable glassy state without crystallizing (Sakai and Engelmann 2007), while encapsulation-dehydration involves embedding explants in alginate beads and gradually dehydrating them before freezing to prevent ice damage (Kulus et al. 2018). Various explants types (shoot tips, nodal segments, buds, zygotic embryos, callus, seeds, and pollen) have been successfully used for cryopreservation (Coste et al. 2012; Kulus 2020; Edesi et al. 2020).

Cryopreservation carries risks, including potential failures during the ultra-freezing or thawing process (Pence et al. 2020). Employing two distinct cryostorage proceeds reduces the chance of complete loss resulting from the breakdown of a single method. This is especially critical for rare and endemic species with limited specimens available for conservation. Given the decline of plant biodiversity, the high risk of extinction, and the potential loss of these endemic plant species, conservation measures are essential for their long-term preservation (González-Benito et al. 1997).

The Romanian flora includes 1,220 species and 201 subspecies mentioned in the national red lists, of which 77 are endemic and 76 subendemic for Romania (Hurdu et al. 2022). Ex situ conservation and cryostorage approaches on endemic plant species from Romania, included in vitro short-term conservation of *Dianthus henteri* (Cristea et al. 2010), micropropagation followed by ex situ outdoor acclimatization in the 'Alexandru Borza' Botanical Garden of the wild species *D. callizonus*, *D. glacialis ssp. gelidus*, and *D. spiculifolius* (Cristea et al. 2013), micropropagation of *Cerastium banaticum* (Cristea et al. 2019), micropropagation of *Astragalus peterfii* (Şuteu et al. 1999), cryopreservation with high regrowth frequencies following cryostorage for seven endemic and endangered *Dianthus* taxa (Halmagyi et al. 2020).

In this study, we build on the foundational work of Halmagyi et al. (2020), extending the application of the

cryostorage approach to address the gap and provide new insights into the conservation of *Moehringia jankae* Griseb. ex Janka - a European endemic species distributed exclusively in the Black Sea region of Romania and Bulgaria. This species is protected by the Bern Convention (1979) and according to the criteria of the European Red List of threatened species, it has been rated as 'Data Deficient' (Bilz et al. 2011). There are few published studies focused on the ex situ conservation of the subendemic taxa *M. jankae*. To date, in Romania, in vitro cultures and medium-term conservation protocols have been developed for this species (Holobiuc et al. 2016, 2018a, b). It is considered vulnerable and rare according to the existing national red lists and is included in the National Law OUG 57/2007 (Annex 3), its protection requiring the designation of special conservation areas (Hurdu et al. 2022). Given its restricted distribution area, specialized biotope, and the combined challenges of climate change adaptation, intensive grazing, and adverse human impacts (Dihoru and Negrean 2009), this species faces significant conservation pressures. Consequently, our study primarily aimed to refine specific cryopreservation protocols to support the ex situ conservation of *M. jankae*. We hypothesize that optimizing sucrose concentration and dehydration duration in the cryopreservation process will significantly improve survival and regeneration rates of *M. jankae* explants following liquid nitrogen storage.

To our knowledge, this study represents the first attempt to optimize cryopreservation protocols for *M. jankae*, and their successful implementation could greatly enhance the long-term preservation of this unique taxon.

Materials and methods

Plant material and micropropagation

The subendemic *Moehringia jankae* Griseb. ex Janka species was selected to be cryopreserved. The plant material used for the induction of in vitro cultures was represented by seeds collected from a Natura 2000 site (Cheia, Constanța County, Romania). Among various disinfection solutions tested the commercial Pursept (with 2.7% sodium dichloroisocyanurate) proved to be the most effective (Holobiuc et al. 2018a). The highest regeneration through axillary shooting was obtained on Murashige and Skoog (1962) (MS) medium with a combination of 6-Benzylaminopurine (BAP) and Indole-3-butyric acid (IBA). Detailed procedures for seed disinfection and the micropropagation protocol have been previously described (Holobiuc et al. 2018a). Figure 1, outlines the steps involved in the cryopreservation of *Moehringia jankae*, from seed collection through to

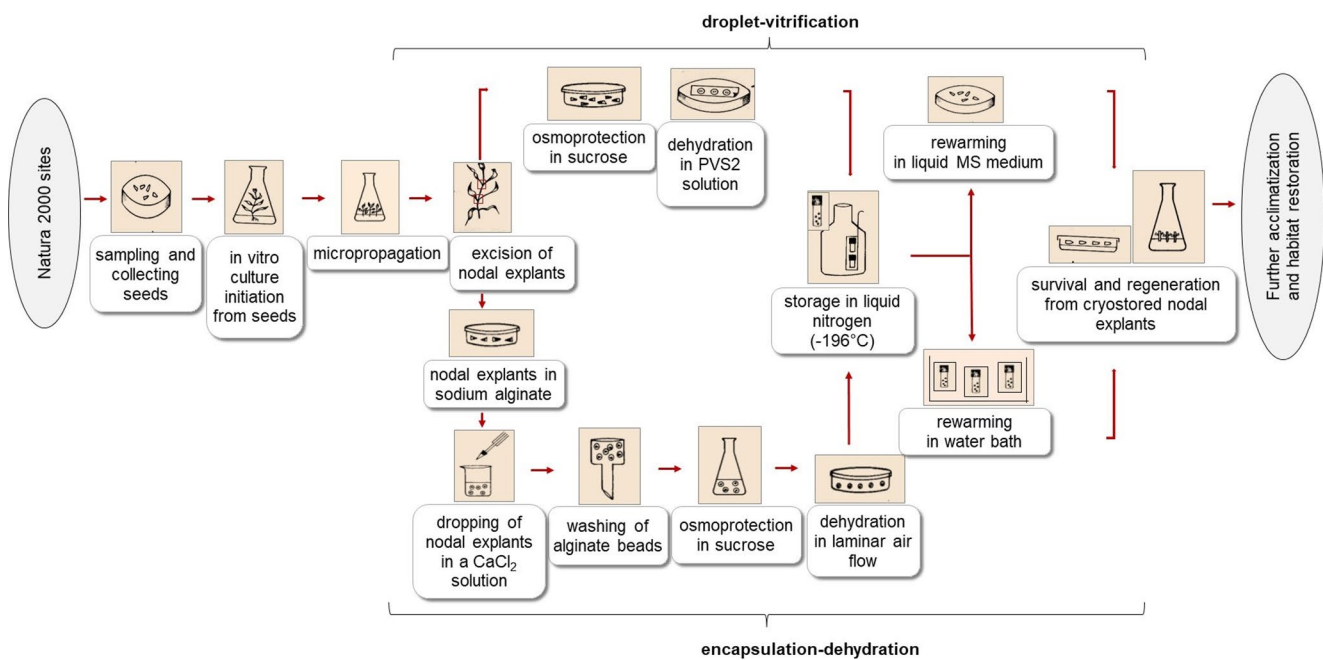


Fig. 1 A comparative workflow of droplet-vitrification and encapsulation-dehydration cryopreservation protocols for *Moehringia jankae* nodal explants

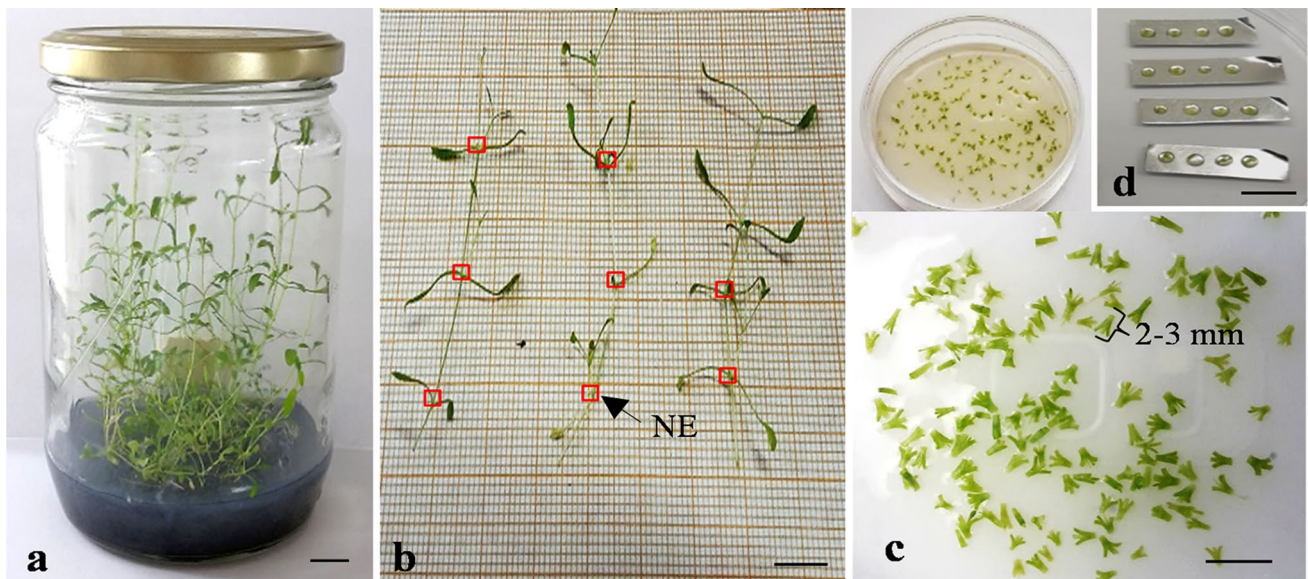


Fig. 2 In vitro culture and preparation of *Moehringia* explants for cryopreservation experiments. (a) in vitro plants, (b) shoots from which nodal explants (red square mark) were excised, (c) nodal explants, (d) nodal explants in PVS2 drops. NE - nodal explants; Scale bar: 1 cm

regeneration after cryostorage by droplet-vitrification and encapsulation-dehydration.

The availability of a substantial number of stock (donor) plants is crucial for the successful implementation of cryopreservation. Thus, for multiplication, apical shoot explants and nodal explants (approximately 2 cm in length) from in vitro plants were transferred to MS (Duchefa) culture medium supplemented with 1.0 mg L⁻¹ *N*6-benzyladenine, (BA) (Sigma) 0.5 mg L⁻¹, Gibberellic acid (GA3)

(Duchefa), 500 mg L⁻¹ activated charcoal (Sigma), 30 g L⁻¹ sucrose and 8 g L⁻¹ agar in glass jars (7 cm diameter and 17 cm height) (Fig. 2a). The pH was adjusted to 5.7 before autoclaving (Systec) (20 min at 121 °C). The density was four explants per jar. The plants were grown at 23 ± 1 °C with a 16-h photoperiod and a light intensity of 40 μmol m⁻² s⁻¹ photosynthetic active radiation provided by white LED (Hepol) lights. The cultures were micropropagated by transferring explants to fresh medium each 30 days. In vitro

multiplied plants were used as explant donors for two cryostorage procedures described further.

Droplet-vitrification (DV) approach

Nodal explants (~2–3 mm in length) were dissected under a stereo microscope in sterile conditions (Fig. 2b). After excision, they were transferred to 5 cm Petri dishes on filter paper moistened with MS liquid medium (without growth regulators) enriched with either 0.25 M, 0.5 M, or 0.75 M sucrose, and incubated for 24 h at each concentration under the same environmental conditions as described above (Fig. 2c). Each sucrose concentration was tested independently, with no sequential transfer between concentrations. For further dehydration the explants were incubated in the plant vitrification solution 2 (PVS2) (Sakai, et al. 1990) for various durations (0, 20, 30, and 40 min) at 23 ± 1 °C. For freezing the explants were individually placed in a drop (6 µl) of PVS2 on previously sterilized (4 h at 160 °C in drying oven) (Binder) aluminium foil strips (0.4 cm x 2.0 cm), transferred to 1.8 ml cryovials (two foils/cryovial) and immersed in liquid nitrogen (−196 °C) contained in a 25 L Dewar flask for 48 h (Fig. 2d).

Encapsulation-dehydration (ED) approach

The excised nodal explants were individually encapsulated in alginate beads by transferring them from a solution of 3% sodium alginate (Duchefa) in Ca^{2+} -free MS to a

solution of 100 mM CaCl_2 and MS mineral salts, where they remained for 20 min under continuous stirring (Fig. 3a–c). After polymerization, the beads (approximately 0.4–0.5 cm in diameter) were washed with liquid MS medium (without CaCl_2) to eliminate excess CaCl_2 (pH 5.7). Encapsulated nodal explants were incubated in MS medium containing sucrose (0.25, 0.50, and 0.75 M) for 24 h on a rotary shaker (GFL) (98 rpm) at 23 ± 1 °C (Fig. 3d). Osmoprotected beads were then dehydrated in laminar air flow (Microflow) up to 4 h at 23 ± 1 °C and a relative humidity of 38% (Fig. 3e, f). At 1-h intervals, dehydrated beads were placed in 1.8 mL cryovials (five beads/cryovial) and immersed in liquid nitrogen.

To maximize the regrowth rates of encapsulated nodal explants after cryopreservation, the water loss from the alginate beads was measured to determine the optimal duration of desiccation. For dry weight (DW) determination, 10 beads were weighed in 3 replications and dried at 60 °C until constant weight was attained. The percentage of moisture content (MC) was calculated after every hour of desiccation and was expressed on a fresh weight (FW) basis using the following formula:

$$\text{MC}(\%) = \frac{\text{FW} - \text{DW}}{\text{FW}} \times 100$$

where FW=fresh weight, DW=dry weight.

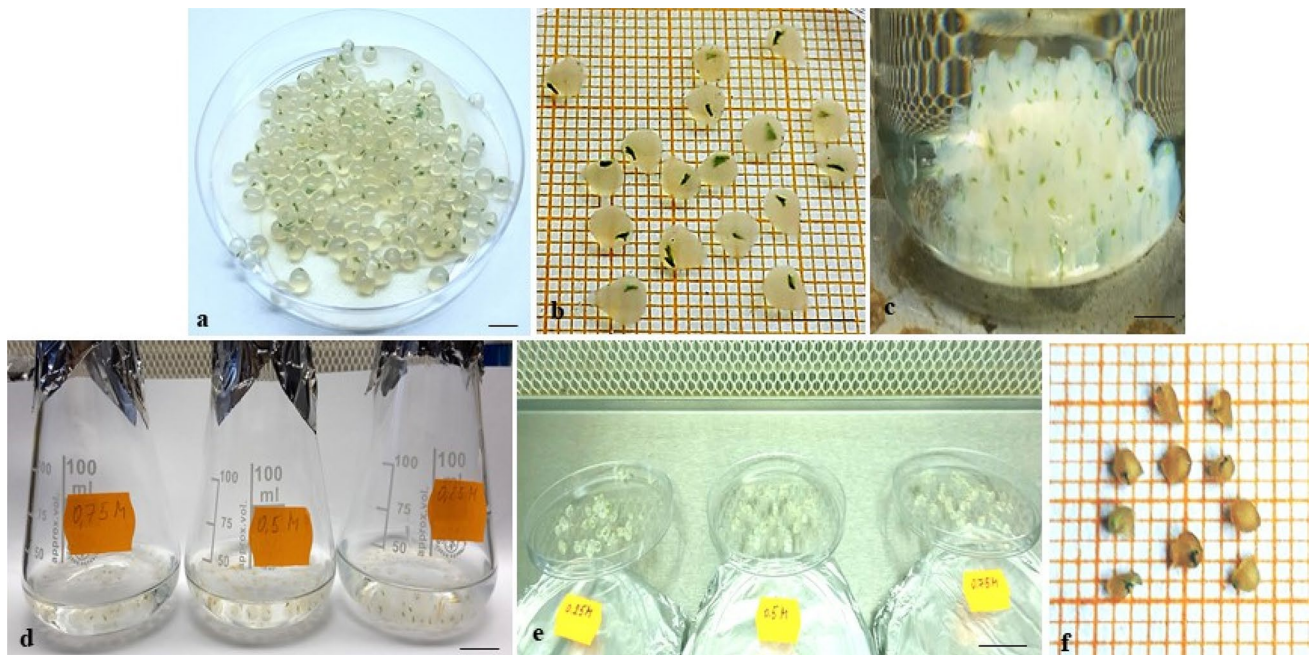


Fig. 3 Encapsulation and dehydration of *Moehringia jankae* nodal explants prior to cryopreservation. (a, b) alginate beads with nodal explants, (c) beads in medium for alginate polymerization, (d) alginate

beads in sucrose solution, (e) desiccation of alginate beads in laminar air flow, (f) desiccated alginate beads. Scale bar: 1 cm

Rewarming

In the DV procedure, after storage in liquid nitrogen, rewarming of samples was performed by quick transfer of the aluminium strips from liquid nitrogen to MS liquid culture medium with 20 g L⁻¹ sucrose (without growth regulators) at room temperature. In the ED procedure, rewarming was performed by immersion of the hermetically closed cryovials in a water bath at 38 °C for 2 min.

Survival and regrowth after cryostorage by DV and ED

For shoot regrowth following cryopreservation (in both procedures), the thawed explants were transferred to MS medium (without growth regulators) with 20 g L⁻¹ sucrose, 6 g L⁻¹ agar in Petri dishes (5 cm in diameter) to regenerate shoots. The growth conditions of explants after freezing for both procedures (DV and ED) were similar to those mentioned for plant multiplication.

Photosynthetic pigment assessment

The chlorophyll (*a*, *b*) and carotenoid content were evaluated in plant material derived from non-cryopreserved (-LN) and cryopreserved (+LN) nodal explants three months post-rewarming for both cryopreservation procedures applied. Three randomly selected shoots were used, with four leaves from the central section of each analyzed shoot. Pigment extraction was conducted using N, N-Dimethylformamide (Wellburn 1994). Quantification was spectrophotometrically (Metertech SP-8001 UV/Visible Spectrophotometer) performed at wavelengths of 664 nm (chlorophyll *a*), 647 nm (chlorophyll *b*), and 480 nm (carotenoids), and was expressed in mg/g fresh weight (FW) (Wellburn 1994) according to the formulas:

$$\text{Chlorophyll } a = 11.65 \times A_{664} - 2.69 \times A_{647} \times v/\text{FW}$$

$$\text{Chlorophyll } b = 20.81 \times A_{647} - 4.53 \times A_{664} \times v/\text{FW}$$

$$\text{Carotenoids} = (1000 \times A_{480} - 0.89 \times \text{chl } a - 52.02 \times \text{chl } b) / 245 \times v/\text{FW}$$

Assessment of recovery and statistical data analyses

The applied cryopreservation procedures followed a completely randomized design. Each cryopreservation treatment was conducted using three replicates, with each replicate consisting of 10 explants. This experimental design allowed a statistically strong comparison of the treatments applied.

The results were expressed as percentages. To comprehensively evaluate the response of nodal explants to cryopreservation, two main parameters were measured: (a) the survival rate, assessed four weeks post-cryopreservation by counting the number of shoots that remained green after storage in liquid nitrogen and reached the early stage of shoot formation (up to 0.3 cm in length), and (b) regrowth, defined as the further development of into shoots with leaf emergence (exceeding 1 cm in length) from the original explants, assessed six weeks after rewarming. Explants that turned brown or white were classified as not surviving the cryo procedure after the same period of time. Survival and regrowth percentages for explants were calculated using the following formulas:

$$\text{Survival (\%)} = \left(\frac{\sum \text{surviving explants}}{\sum \text{number of total explants}} \right) \times 100$$

$$\text{Regrowth (\%)} = \left(\frac{\sum \text{regrowing explants}}{\sum \text{number of total explants}} \right) \times 100$$

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Duncan's honestly significant difference (HSD) test ($PB \leq 0.05$) using SPSS program ver. 17.0 (SPSS Inc., Chicago, USA, 2008).

Results

Survival and regrowth following DV

As cryopreservation studies have not been conducted on this species, first we had to test the survival and regrowth capacity of nodal explants after osmoprotection and dehydration without liquid nitrogen storage. Survival and regrowth of nodal explants after 24 h treatment in sucrose slightly decreased (non-significantly) at higher sucrose concentrations, showing 93% survival and shoot regrowth at 0.75 M sucrose (Table 1). In contrast, the addition of PVS2 dehydration treatment for 15 min significantly affected shoot survival and regrowth. At no sucrose, survival was 36% and regrowth was 28%, while at 0.5 M sucrose, both survival and regrowth reached 95%. At a higher sucrose concentration (0.75 M) survival significantly decreased to 80% and regrowth to 75% (Table 1).

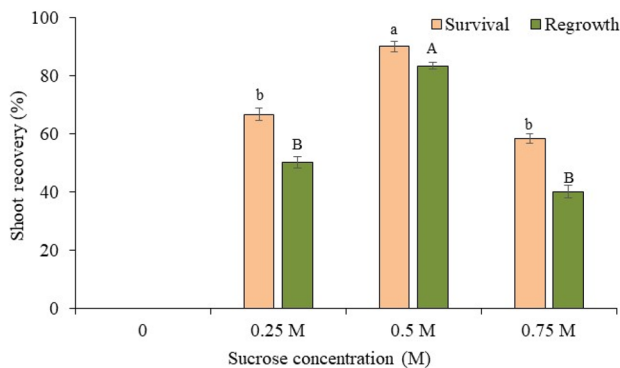
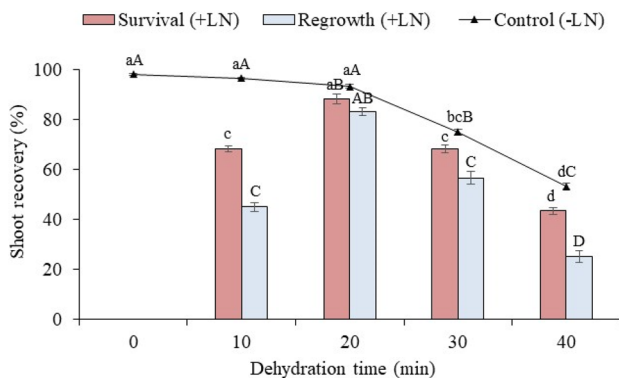
Shoot tips that survived cryostorage turned green and began sprouting around 10–12 days after rewarming. In contrast, non-surviving shoot tips turned dark brown or white, exhibited necrosis with no visible signs of recovery.

Examining the impact of various sucrose concentrations on survival and regrowth after liquid nitrogen storage, the key findings are as follows: the maximum survival (90%)

Table 1 Explant responses to osmoprotection and PVS2 dehydration treatments

		Shoot development (% $\pm$ SD)*			
		Sucrose concentration (M)			
		0	0.25	0.5	0.75
Control	Survival	100 ^a	100 ^a	98.3 $\pm$ 0.4 ^a	93.3 $\pm$ 1.0 ^{ab}
	Regrowth	100 ^A	98.3 $\pm$ 0.4 ^A	96.6 $\pm$ 0.5 ^A	93.3 $\pm$ 0.8 ^A
Non-cryopreserved treatment	Survival	36.6 $\pm$ 3.0 ^c	75.0 $\pm$ 2.8 ^b	95.0 $\pm$ 0.5 ^{ab}	80.0 $\pm$ 2.0 ^b
	Regrowth	28.3 $\pm$ 1.1 ^C	75.0 $\pm$ 1.0 ^B	95.0 $\pm$ 0.8 ^A	75.0 $\pm$ 1.0 ^B

*Values represent means $\pm$ standard deviation (SD). Control refers to osmoprotection in sucrose for 24 h; Non-cryopreserved treatment refers to osmoprotection in sucrose (24 h) followed by 15 min PVS2 dehydration. Values followed by different letters within rows indicate significant differences ($p \leq 0.05$): lower-case letters represent significant differences between survival of non-cryopreserved treatment vs. control, and different capital letters indicate significant differences between regrowth of non-cryopreserved treatment vs. control

**Fig. 4** Shoot survival and regrowth from osmoprotected, dehydrated and cryopreserved nodal explants. Treatments involved incubation in sucrose (24 h), dehydration in PVS2 (15 min) and freezing in liquid nitrogen. Bars represent the standard deviation. Different letters indicate significant differences ($p \leq 0.05$): lower-case letters represent significant differences among survival variants, while different capital letters indicate significant differences among regrowth variants**Fig. 5** Impact of PVS2 dehydration duration on post-thaw survival and regrowth following cryostorage. Bars represent the standard deviation. Different letters indicate significant differences ($p \leq 0.05$): lower-case letters represent significant differences between survival variants vs. control, and different capital letters indicate significant differences between regrowth variants vs. control

and the highest regrowth rate (83%) occurred at the same sucrose concentration (0.5 M). However, higher concentrations of sucrose such as 0.75 M led to a significant decline in both parameters (Fig. 4). As the highest regrowth efficiency was achieved at 0.5 M sucrose concentration, only this concentration was used in the subsequent experiments. Figure 4 presents the survival and regrowth rates across different PVS2 exposure durations, comparing conditions with liquid nitrogen (+LN) and without it (-LN). Non-cryopreserved (-LN) nodal explants showed high survival rates across all exposure times, although survival decreased with longer exposure. However, explants that were not treated with PVS2 exhibited no recovery.

The highest survival rate following freezing was 88% and was recorded for 20 min PVS2 exposure. Beyond 20 min, survival decreased to 43% at 40 min of dehydration treatment. Regrowth of nodal explant buds mirrored survival trends. At no dehydration, regrowth was absent, while the maximum regrowth was 83% and occurred after 20 min PVS2 exposure followed by a decline to 25% at 40 min dehydration (Fig. 5). Noticeable differences were observed between treatments. Abundant healthy shoots appeared after 0.25 M sucrose (Fig. 6a), while fewer shoots followed 0.25 M sucrose + 20 min PVS2 dehydration (Fig. 6b). Cryopreservation resulted in limited regrowth and several non-viable explants (red-circled) (Fig. 6c). Similar healthy shoots were seen after 0.5 M sucrose treatment (Fig. 6d), with a slight reduction after PVS2 dehydration (Fig. 6e). Few shoots survived cryopreservation (Fig. 6f).

Survival and regrowth following ED

For the same reason as mentioned earlier, we considered it necessary to test the survival and regenerative capacity of the nodal explants after encapsulation in alginate beads without freezing in liquid nitrogen. Survival and regrowth of nodal explants after encapsulation in alginate beads and 24 h sucrose osmoprotection treatment (without cryopreservation) was high, with a non-significant decrease at higher

Fig. 6 Shoot regrowth from nodal explants after the droplet vitrification procedure. **(a)** 0.25 M sucrose, control, **(b)** 0.25 M sucrose+20 min PVS2 dehydration, control, **(c)** 0.25 M sucrose+20 min PVS2 dehydration, cryopreserved, **(d)** 0.5 M sucrose, control, **(e)** 0.5 M sucrose+20 min PVS2 dehydration, control, **(f)** 0.5 M sucrose+20 min PVS2 dehydration, cryopreserved. The red-circled nodal explants represent non-viable tissues following cryopreservation. Scale bar: 1 cm

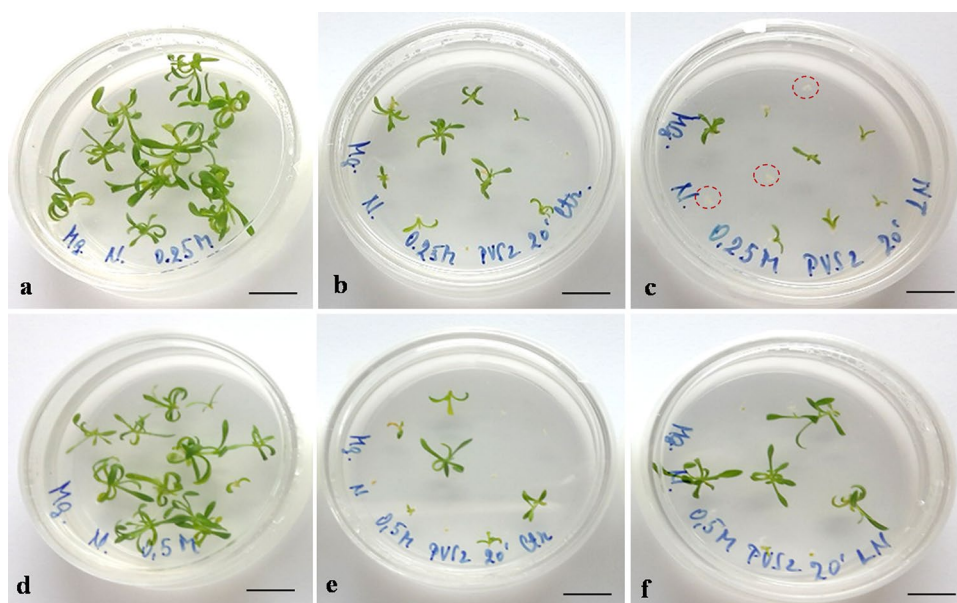


Table 2 Nodal explant responses to encapsulation and sucrose osmoprotection treatments

		Shoot development (% $\pm$ SD)*			
		Sucrose concentration (M)			
Encapsulated nodal explants + sucrose (24 h)	Survival	100 ^a	100 ^a	98.3 $\pm$ 0.4 ^a	98.3 $\pm$ 0.4 ^a
	Regrowth	100 ^A	98.3 $\pm$ 0.4 ^A	98.3 $\pm$ 0.4 ^A	96.6 $\pm$ 0.8 ^A

*Values represent means $\pm$ standard deviation (SD). Values followed by different letters within a row indicate significant differences ($p \leq 0.05$): lower-case letters represent significant differences between survival variants, and different capital letters indicate significant differences between regrowth variants

sucrose concentrations (0.75 M), showing 98% survival and 96% shoot regrowth (Table 2).

Following cryopreservation no shoot survival was obtained at no sucrose osmoprotection (Fig. 7a). It was observed that survival rates and shoot regrowth showed a similar trend, respectively, an increase with higher desiccation durations for all sucrose concentrations tested (Fig. 7b–d). The highest survival (90%) and regrowth (85%) were reached after osmoprotection treatment in 0.75 M and 4 h desiccation (Fig. 7d). The highest regrowth (85%) at 0.75 M sucrose was correlated with 23% water content of alginate beads (Figs. 7d and 8).

Our results revealed that there were no significant differences between the two methods (DV and ED) when comparing the highest regrowth rates. An average survival rate of 88% and a regrowth rate of 83% were achieved after 24 h osmoprotection in 0.5 M sucrose and 20 min PVS2 dehydration using the droplet vitrification method, while the encapsulation-dehydration approach yielded the highest survival (90%) and regrowth (85%) following osmoprotection with 0.75 M sucrose and 23% water content of alginate beads.

Photosynthetic pigment content

Chlorophyll *a*, chlorophyll *b*, and carotenoid levels in plants resulting from cryopreserved nodal explants did not differ significantly from those in plants derived from non-cryopreserved explants. The data indicate that the applied cryopreservation methods had no significant impact on these pigment levels (Table 3). Although carotenoid concentrations were slightly higher in control (–LN) plants, this difference was not statistically significant across both cryopreservation procedures (Table 3).

Discussion

The success of cryostorage relies on maintaining the viability and regenerative capacity of the preserved plant material (Halmagyi et al. 2020). In vitrification-based procedures, to prevent cell damage caused by the formation of ice crystals at ultra-low freezing temperatures, explants are treated with highly concentrated cryoprotectant solutions prior to freezing. Among these, PVS2 is the most widely used cryoprotectant across many plant species (Wang et al. 2014). The cytotoxicity of PVS2 varies significantly depending

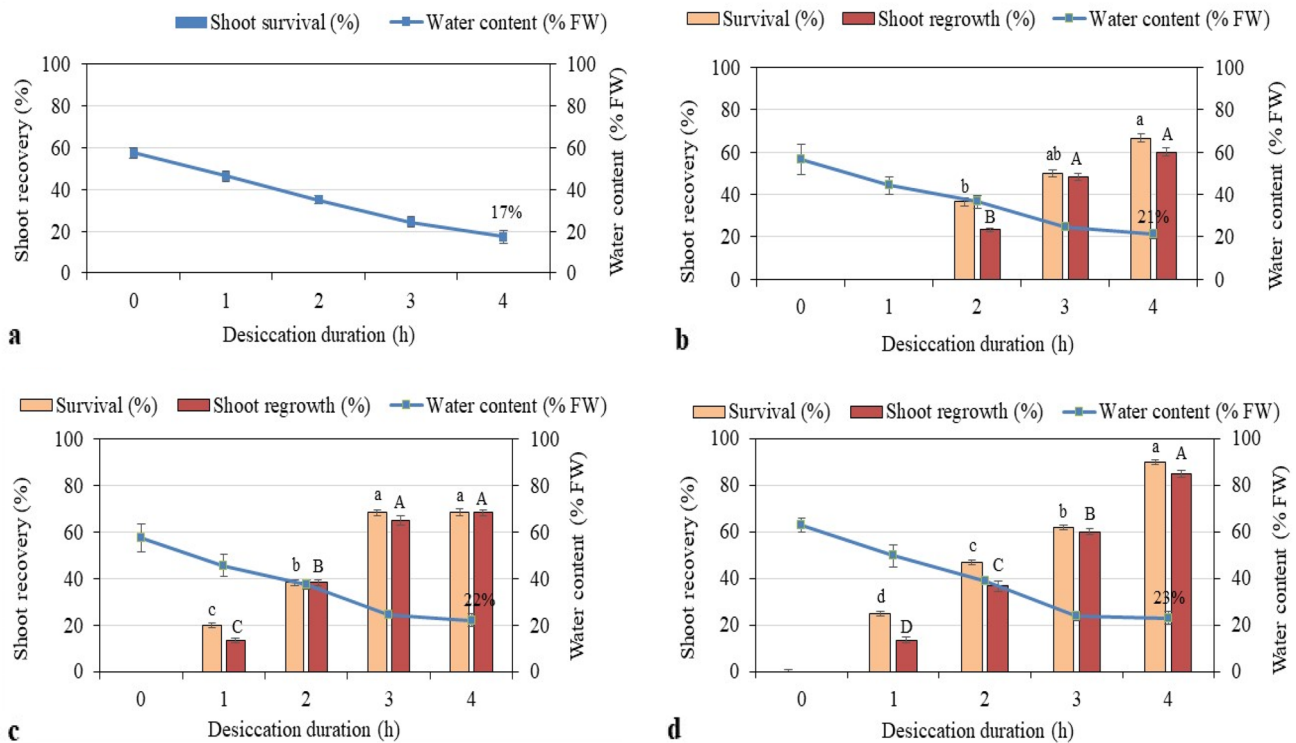


Fig. 7 Survival and regrowth of nodal explants after liquid nitrogen storage based on the moisture content of alginate beads. (a) no sucrose, (b) 0.25 M sucrose, (c) 0.5 M sucrose, (d) 0.75 M sucrose. Bars represent the standard deviation. Different letters indicate significant differences ($p \leq 0.05$): lower-case letters represent significant differences among survival variants, and different capital letters indicate significant differences among regrowth variants

on the species and even a specific genotype (Kim 2023). It was reported that preculture in sucrose improved tolerance to both cryoprotection and cryopreservation by acquiring dehydration and freezing tolerance (Kim et al. 2010). The successful recovery of cryopreserved samples also relies on determining the optimal PVS2 treatment duration that effectively balances cryoprotectant toxicity with the need for adequate dehydration (Panis et al. 2009). Therefore, in the present study, we assessed the effects of various parameters such as sucrose concentration, PVS2 dehydration duration (in DV), moisture content of alginate beads, sucrose concentration and desiccation duration (in ED) on post-thaw survival and regrowth of *M. jankae* nodal explants following liquid nitrogen storage.

Our results revealed that there were no significant differences between the two methods (DV and ED) when comparing the highest regrowth rates. An average survival rate of 88% and a regrowth rate of 83% were achieved after 24 h osmoprotection in 0.5 M sucrose and 20 min PVS2 dehydration using the droplet vitrification method, while the encapsulation-dehydration approach yielded the highest survival (90%) and regrowth (85%) following osmoprotection with 0.75 M sucrose and 23% water content of alginate beads.

Notably, variations in post-cryopreservation regrowth rates have been observed in different species. For instance *Garcinia indica* (an endemic species for India) showed the highest (78%) explant regrowth after applying the droplet vitrification procedure and 10 min PVS2 exposure (Srivastava et al. 2024). The use of 30 min PVS2 dehydration led to 44% post-thaw recovery for *Dahlia* shoot tips (Gowthami et al. 2023). Van der Walt et al. (2023) reported that the use of a novel metal-mesh vacuum infiltration vitrification method based on 20 min PVS2 treatment resulted in 19% post-cryopreservation survival for *Syzygium maire* (critically endangered tree species endemic to New Zealand). For *Draba yukonensis* (endemic plant species for Canada), the optimal PVS3 treatment period was 30 min exposure, which resulted in an average regrowth of 67% (Saxena et al. 2021).

In comparison to the results presented for other endemic species in our study, we obtained high (83%) regrowth rates for *M. jankae* nodal explants after applying the DV procedure. It was reported that the PVS2 solution exhibited toxicity in the absence of sucrose preculture (Halmagyi and Pinker 2006). For *M. jankae*, a sucrose (0.5 M) treatment for 24 h followed by PVS2 dehydration treatment (20 min) contributed to a reduced water content in cells by creating an osmotic gradient, which minimizes ice crystal formation during freezing, preventing cellular damage. In the absence

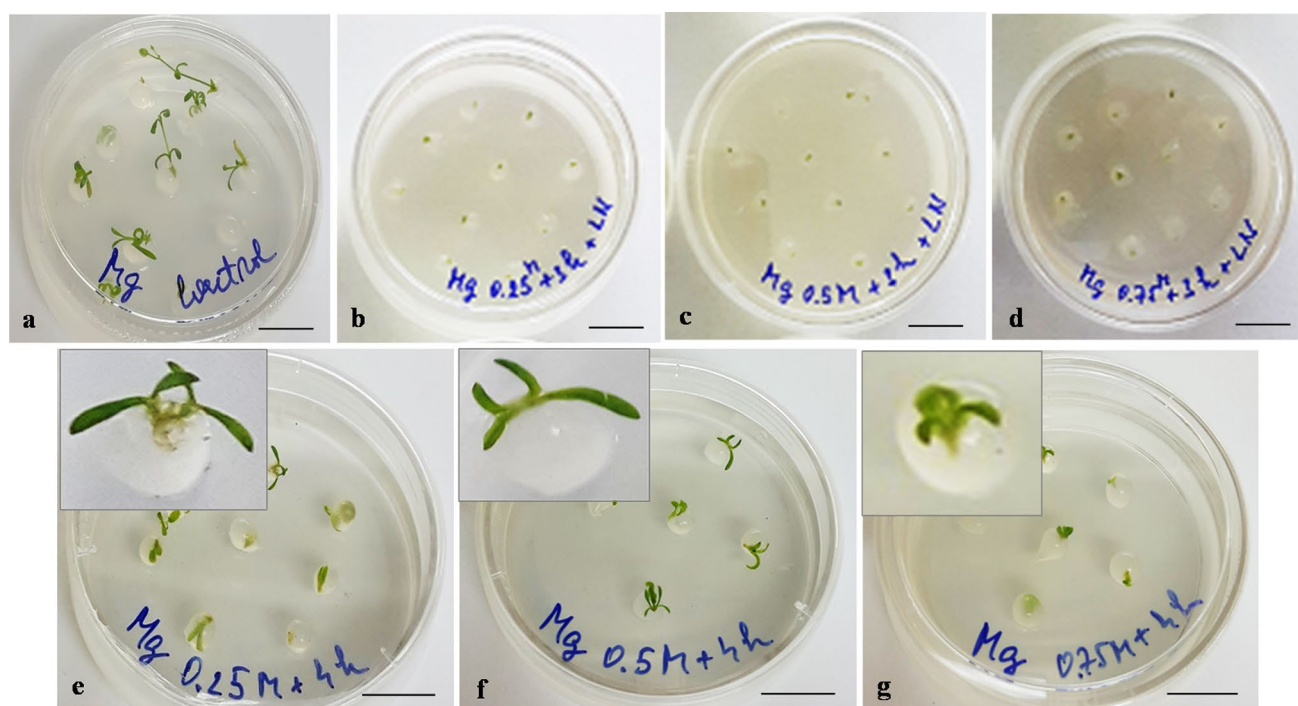


Fig. 8 Shoot regrowth from nodal explants after the encapsulation-dehydration procedure. (a) encapsulated control nodal explants, (b) osmoprotection in 0.25 M sucrose+3 h desiccation and cryopreservation, (c) osmoprotection in 0.5 M sucrose+3 h desiccation and cryopreservation, (d) osmoprotection in 0.75 M sucrose+3 h desiccation

and cryopreservation, (e) osmoprotection in 0.25 M sucrose+4 h desiccation and cryopreservation, (f) osmoprotection in 0.5 M sucrose+4 h desiccation and cryopreservation, (g) osmoprotection in 0.25 M sucrose+4 h desiccation and cryopreservation, Scale bar: 1 cm

Table 3 Chlorophyll and carotenoid content in leaves originating from non-cryopreserved (–LN) and cryopreserved (+LN) nodal explants following DV and ED

Procedure		Chlorophyll a (mg/g FW±SD)*	Chlorophyll b (mg/g FW±SD)*	Carotenoids (mg/g FW±SD)*
DV	–LN	0.84±0.07 ^a	0.35±0.02 ^b	0.06±0.01 ^c
	+LN	0.85±0.09 ^a	0.38±0.18 ^b	0.04±0.00 ^c
ED	–LN	0.86±0.11 ^A	0.38±0.05 ^B	0.08±0.01 ^C
	+LN	0.83±0.09 ^A	0.36±0.05 ^B	0.06±0.02 ^C

*Values represent means±standard deviation (SD). Values followed by the same letter within rows and variants are not significantly different ($p \leq 0.05$); lower-case letters represent significant differences between DV variants, and different capital letters indicate significant differences between ED variants

of sucrose osmotic dehydration, no survival was obtained for *M. jankae*. Sucrose, like many other sugars, is widely recognized for its crucial role in enhancing the desiccation resistance of plant tissues and organs (Dumet et al. 1993).

Encapsulation-dehydration exhibited wide survival rate limits in other endemic species, similar to the variations observed with DV. In the case of ED, the water content of the alginate beads played a major role in achieving high regrowth rates after cryopreservation. ED has been less commonly used for endemic or rare plants compared to other cryostorage methods. For instance, up to 48% post-cryopreservation survival was obtained for *Cyathea australis* (prominent component of some threatened forest communities) spores after encapsulation and 5 h dehydration (Mikuła et al. 2009). In *Ceratopetalum gummiferum* (endemic species in Australia) encapsulated shoot tips desiccated at 24%

moisture content (6 h), the regrowth after cryopreservation was 61% (Shatnawi and Johnson 2004). Encapsulation-dehydration led to 48% shoot regrowth for *Viola stagnina* (endangered species of wet habitats) (Żabicki et al. 2021).

Encapsulation-dehydration was an efficient technique for the cryopreservation of *M. jankae* nodal explants, showing the highest survival (90%) and recovery (85%) following osmoprotection with 0.75 M sucrose and 23% water content of alginate beads after 4 h of desiccation. González-Benito et al. (1997) reported 70% survival for *Centaurea rigualii* (an endemic endangered plant species of the Iberian Peninsula) encapsulated nodes at a moisture content of 27% reached after 4 h desiccation. A high percentage (63%) with the encapsulation-dehydration method was also achieved for a rare endemic species in Portugal, *Plantago algarbiensis*, after 3 h desiccation (Coelho et al. 2014b).

Encapsulation-dehydration gave 50% shoot tip survival for *Thymus moroderi*, an endemic plant from Spain, after cold-hardening of donor plants, preculture (for 48 h), and 22% moisture content of alginate beads (Marco-Medina et al. 2010). A 24% water content of alginate beads led to 69% recovery for apple shoot tips after encapsulation-dehydration (Butiuc-Keul et al. 2019). In *M. jankae*, shoots were regenerated directly from nodal explants without callus formation in both cryostorage approaches tested. Callus formation is generally considered undesirable in plant germplasm conservation due to the potential occurrence of somaclonal variation (Larkin and Scowcroft 1981).

Conclusions

Moehringia nodal explants exhibited strong regrowth after cryostorage using both methods (DV and ED). The high regeneration rates demonstrate that *Moehringia* is not a sensitive species to the treatments applied prior cryopreservation, nor to the effects of liquid nitrogen on the tissues. When comparing the two techniques (DV and ED), there was no significant difference between the best variants in terms of survival and regrowth following cryostorage. A long-term goal is to apply these cryopreservation methods to other endemic plant species, particularly those facing threats from habitat destruction, climate change, and human activity. Furthermore, increasing awareness and encouraging sustainable practices are essential for ensuring the long-term preservation of endemic species.

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Author contributions A.H. designed and performed the cryopreservation experiments and wrote the paper. A.C. contributed to the cryopreservation experiments, in vitro micropropagation and performed the statistical analysis of the data. All authors read and approved the final manuscript.

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Data availability All experimental data and pictures are available at the first author.

Declarations

Competing Interests The authors (Halmagyi, A. and Coste, A.) have no relevant financial or non-financial interests to disclose.

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References

- Benelli C, Tarraf W, Izgü T, Anichini M et al (2024) Long-term conservation for the safeguard of *Abies nebrodensis*: an endemic and endangered species of Sicily. *Plants* 13:1682. <https://doi.org/10.3390/plants13121682>
- Bern Convention (1979) Convention on the Conservation of European Wildlife and natural Habitats. Appendix I. <https://rm.coe.int/CoERMPublicCommonSearchServices/DisplayDCTMContent?documentId=0900001680304354>, 1979. Accessed 28 January 2025
- Bilz M, Kell SP, Maxted N, Lansdown RV (2011) European Red List of Vascular Plants. Luxembourg
- Butiuc-Keul A, Coste A, Farkas A, Cristea V, Isac V, Halmagyi A (2019) Molecular characterization of Apple (*Malus x domestica* Borkh.) genotypes originating from three complementary conservation strategies. *Turkish J Agric Forestry* 43:464–477. <https://doi.org/10.3906/tar-1803-3>
- Coelho N, González-Benito ME, Romano A (2014a) Cryopreservation of shoot tips from the endangered endemic species *Tuberaria major*. *Acta Physiol Plant* 36:333–336. <https://doi.org/10.3390/plants9030345>
- Coelho N, González-Benito ME, Romano A (2014b) Approaches for the cryopreservation of *Plantago algarbiensis*, a rare endemic species of the Algarve. *CryoLetters* 35:521–529.
- Coelho N, Gonçalves S, Romano A (2020) Endemic plant species conservation: biotechnological approaches. *Plants* 9:345. <https://doi.org/10.3390/plants9030345>
- Coste A, Halmagyi A, Butiuc-Keul AL, Deliu C, Coldea G, Hurdu B (2012) In vitro propagation and cryopreservation of Romanian endemic and rare *Hypericum* species. *Plant Cell Tiss Org Cult* 110:213–226. <https://doi.org/10.1007/s11240-012-0144-7>
- Cristea V, Brummer AT, Jarda L, Miclăuş M (2010) In vitro culture initiation and phytohormonal influence on *Dianthus henteri* - a Romanian endemic species. *Rom Biotechnol Lett Suppl* 15:25
- Cristea V, Jarda L, Holobiuc I (2013) Ex situ conservation of three endemic and/or endangered *Dianthus* species. *Not Bot Horti Agro* 41:73–78. <https://doi.org/10.15835/nbha4119110>
- Cristea V, Besenyei E, Jarda L, Farkas A, Marcu D, Clapa D, Halmagyi A, Butiuc-Keul A (2019) In situ genetic variability and micropropagation of *Cerastium banaticum* (Rochel) heuff. (Caryophyllaceae) - a rare and endemic species from Romania. *Acta Biol Cracov S Bot* 61:65–74. <https://doi.org/10.24425/abcsb.2019.127737>
- Dihoru G, Negrean G (2009) Cartea Roşie a Plantelor vasculare Din România. Editura Academiei Române
- Dumet D, Engelmann F, Chabrilange N, Duval Y (1993) Cryopreservation of oil palm (*Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. *Plant Cell Rep* 12:352–355. <https://doi.org/10.1007/BF00237434>

- Edesi J, Tolonen J, Ruotsalainen AL, Aspi J, Häggman H (2020) Cryopreservation enables long-term conservation of critically endangered species *Rubus humilifolius*. *Biodivers Conserv* 29:303–314. <https://doi.org/10.1007/s10531-019-01883-9>
- Engels JMM, Ebert AW (2021) A critical review of the current global ex situ conservation system for plant agrobiodiversity. Part II. Strengths and weaknesses of the current system and recommendations for its improvement. *Plants* 10:1904. <https://doi.org/10.3390/plants10091904>
- Gonçalves-Souza D, Verburg PH, Dobrovolski R (2020) Habitat loss, extinction predictability and conservation efforts in the terrestrial ecoregions. *Biol Conserv* 246:108579. <https://doi.org/10.1016/j.biocon.2020.108579>
- González-Benito MA, Pérez C, Viviani AB (1997) Cryopreservation of nodal explants of an endangered plant species (*Centaurea rigualii* Esteve) using the encapsulation-dehydration method. *Biodivers Conserv* 6:583–590. <https://doi.org/10.1023/A:1018337429589>
- Gowthami R, Chander S, Pandey R, Shankar M, Agrawal A (2023) Development of efficient and sustainable droplet-vitrification cryoconservation protocol for shoot tips for long-term conservation of *Dahlia* germplasm. *Sci Hortic* 321:112329. <https://doi.org/10.1016/j.scienta.2023.112329>
- Halmagyi A, Pinker I (2006) Plant regeneration from *Rosa* shoot tips cryopreserved by a combined droplet vitrification method. *Plant Cell Tiss Org Cult* 84:145–153. <https://doi.org/10.1007/s11240-005-9012-z>
- Halmagyi A, Coste A, Tripon S, Craciun C (2017) Low temperature induced alterations in tomato (*Lycopersicon esculentum* Mill.) shoot apex cells. *Sci Hortic* 222:22–31. <https://doi.org/10.1016/j.scienta.2017.04.019>
- Halmagyi A, Coste A, Jarda L, Butiuc-Keul A, Holobiuc I, Cristea V (2020) A safeguard measure of endemic and endangered plant species: cryostorage of *Dianthus* taxa. *Biodivers Conserv* 29:3445–3460. <https://doi.org/10.1007/s10531-020-02032-3>
- Heinken T, Weber E (2013) Consequences of habitat fragmentation for plant species: do we know enough? Perspectives in plant. *Ecol Evol Syst* 15:205–216. <https://doi.org/10.1016/j.ppees.2013.05.003>
- Heywood VH (2019) Conserving plants within and beyond protected areas - still problematic and future uncertain. *Plant Divers* 41:36e49. <https://doi.org/10.1016/j.pld.2018.10.001>
- Holobiuc I, Catană R, Maximilian C, Anastasiu P, Cristea V (2016) *In vitro* culture for ex situ conservation of the vulnerable species *Moehringia Janke* Griseb ex Janka. *Studia Univ Babeş-Bolyai Biol LXI*:35–36
- Holobiuc I, Catană R, Cogălniceanu G, Cristea V (2018a) Biotechnological approach for ex situ conservation of the vulnerable species *Moehringia Jankae*. *Rom Biotechnol Lett* 23:13954–13963
- Holobiuc I, Catană R, Maximilian C, Cristea V, Mitoi ME (2018b) Ex situ conservation using medium-term cultures in *Moehringia Jankae* griseb. Ex Janka (Caryophyllales: Caryophyllaceae) and genetic stability assessment using ISSR. *Acta Zool Bulg Suppl* 11:155–162
- Hurdu BI, Coste A, Halmagyi A et al (2022) Ex situ conservation of plant diversity in Romania: a synthesis of threatened and endemic taxa. *J Nat Conserv* 68:126211. <https://doi.org/10.1016/j.jnc.2022.126211>
- Kim H (2023) Optimizing the droplet-vitrification procedure by balancing the cryoprotection and cytotoxicity of alternative plant vitrification solutions based on the nature of donor plant vigor. *Plants* 12:4040. <https://doi.org/10.3390/plants12234040>
- Kulus D (2019) Application of synthetic seeds in propagation, storage, and preservation of Asteraceae plant species. In: Faisal M, Alatar A (eds) *Synthetic seeds*. Springer, Cham, pp 155–179
- Kulus D (2020) Cryopreservation of bleeding heart (*Lamprocapnos spectabilis* (L.) Fukuhara) shoot tips using encapsulation-dehydration. *Cryoletters* 41:75–85
- Kulus D, Zalewska M (2014) Cryopreservation as a tool used in long-term storage of ornamental species - a review. *Sci Hortic* 168:88–107. <https://doi.org/10.1016/j.scienta.2014.01.014>
- Kulus D, Abratowska A, Mikula A (2018) Morphogenetic response of shoot tips to cryopreservation by encapsulation-dehydration in a solid mutant and periclinal chimeras of *Chrysanthemum x grandiflorum* /Ramat./Kitam. *Acta Physiol Plant* 40:18–31. <https://doi.org/10.1007/s11738-017-2593-4>
- Kulus D, Tymoszuk A, Kulpińska A et al (2025) Synergistic effects of iron oxide nanoparticles and indole-3-acetic acid on the germination and development of cold-stored chrysanthemum synthetic seeds. *Plant Cell Tissue Organ Cult* 160:18. <https://doi.org/10.1007/s11240-024-02955-7>
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197–214. <https://doi.org/10.1007/BF02342540>
- Manes S, Costello MJ, Beckett H, Debnath A et al (2021) Endemism increases species' climate change risk in areas of global biodiversity importance. *Biol Conserv* 257:109070. <https://doi.org/10.1016/j.biocon.2021.109070>
- Marchese C (2015) Biodiversity hotspots: A shortcut for a more complicated concept. *Global Ecol Conserv* 3:297–309. <https://doi.org/10.1016/j.gecco.2014.12.008>
- Marco-Medina A, Casas JL, Gonzalez-Benito ME (2010) Comparison of vitrification and encapsulation-dehydration for cryopreservation of *Thymus moroderi* shoot tips. *CryoLetters* 31:301–319
- Martín C, Šajna N, Kaligarič M, Ambrožič-Dolinšek J (2018) Cryopreservation of an endangered *Hladnikia pastinacifolia* Rchb. by shoot tip encapsulation-dehydration and encapsulation-vitrification. *In Vitro Cell Dev Biol* 54:565–575. <https://doi.org/10.1007/s11627-018-9917-y>
- Mikula A, Jata K, Rybczyński JJ (2009) Cryopreservation strategies for *Cyathea australis* (R. BR.) DOMIN. *CryoLetters* 30:429–439
- Mkala EM, Mwanzia V, John Nzei J, Oluoch WA et al (2023) Predicting the potential impacts of climate change on the endangered endemic Annonaceae species in East Africa. *Heliyon* 9:e17405. <https://doi.org/10.1016/j.heliyon.2023.e17405>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cell cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nunez S, Arets E, Alkemade R, Verwer C, Leemans R (2019) Assessing the impacts of climate change on biodiversity: is below 2°C enough? *Clim Change* 154:351–365. <https://doi.org/10.1007/s10584-019-02420-x>
- Panis B, Piette B, Andre E, Van den Houwe I, Swennen R (2009) Droplet vitrification: the first generic cryopreservation protocol for organized plant tissues? *Acta Hortic* 908:157–164
- Pence VC, Ballesteros D, Walters C, Reed BM, Philpott M, Dixon KW, Pritchard HW, Culley TM, Vanhove A C (2020) Cryobiotechnologies: Tools for expanding long-term ex situ conservation to all plant species. *Biol Conserv* 250:108736. <https://doi.org/10.1016/j.biocon.2020.108736>
- Ruta C, Lambardi M, Ozudogru EA (2020) Biobanking of vegetable genetic resources by *in vitro* conservation and cryopreservation. *Biodivers Conserv* 29:3495–3532
- Sakai A, Engelmann F (2007) Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *CryoLetters* 28:151–172
- Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* osb. Var. *Brasiliensis* Tanaka) by vitrification. *Plant Cell Rep* 9:30–33. <https://doi.org/10.1007/BF00232130>

- Saxena A, Bi WL, Shukla MR, Cannings S, Bennett B, Saxena PK (2021) Micropropagation and cryopreservation of Yukon Draba (*Draba yukonensis*), a special concern plant species endemic to Yukon territory, Canada. *Plants* 10:2093. <https://doi.org/10.3390/plants10102093>
- Schrader J, Weigelt P, Cai L, Westoby M et al (2024) Islands are key for protecting the world's plant endemism. *Nature* 634:868–874. <https://doi.org/10.1038/s41586-024-08036-1>
- Shatnawi MA, Johnson KA (2004) Cryopreservation by encapsulation–dehydration of ‘christmas bush’ (*Ceratopetalum Gummiferum*) shoot tips. *In Vitro Cellular & Developmental Biology* 40:239–244. <https://doi.org/10.1079/IVP2003518>
- SPSS Inc Released 2008. SPSS statistics for windows, version 17.0. Chicago: SPSS Inc
- Srivastava V, Panis B, Agrawal A (2024) Droplet vitrification: a lifeline for longterm conservation of threatened species *Garcinia indica*. *Vitro Cell Dev Biol Plant* 60:318–332. <https://doi.org/10.1007/s11627-024-10423-2>
- Staude IR, Navarro LM, Pereira HM (2020) Range size predicts the risk of local extinction from habitat loss. *Glob Ecol Biogeogr* 29:16–25. <https://doi.org/10.1111/geb.13003>
- Şuteu A, Butiuc-Keul A, Mocan S, Pârvu M (1999) Researches concerning *in vitro* micropropagation of *Astragalus Peterfii* Jáv., an endangered species of the Romanian flora. *Contrib Bot* 2:209–213
- Van der Walt K, Nadarajan J, Mathew L, Bettoni JC, Souza JA (2023) Advances in cryopreservation of *Syzygium Maire* (swamp Maire, Maire tawake) zygotic embryos, a critically endangered tree species endemic to new Zealand. *Front Conserv Sci* 4:1269881. <https://doi.org/10.3389/fcsc.2023.1269881>
- Wang B, Li JW, Zhang ZB, Wang RR, Ma YL, Blystad DR, Keller ERJ, Wang QC (2014) Three vitrification-based cryopreservation procedures cause different cryo-injuries to potato shoot tips while all maintain genetic integrity in regenerants. *J Biotechnol* 184:47–55. <https://doi.org/10.1016/j.jbiotec.2014.04.021>
- Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using solvents with spectrophotometers of different resolution. *J Plant Physiol* 144:307–313. [https://doi.org/10.1016/S0176-1617\(11\)81192-2](https://doi.org/10.1016/S0176-1617(11)81192-2)
- Żabicki P, Mikula A, Sliwinska E, Migdalek G, Nobis A, Żabicka J, Kuta E (2021) Cryopreservation and post-thaw genetic integrity of *Viola Stagnina* kit., an endangered species of wet habitats - A useful tool in ex situ conservation. *Sci Hortic* 284:110056. <https://doi.org/10.1016/j.scienta.2021.110056>

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