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A promising strategy for conservation of Central Asian endemic plant *Euonymus koopmannii*

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Abstract. *Gubaidullin N, Kali B, Tussipkan D, Manabayeva SA. 2024. A promising strategy for conservation of Central Asian endemic plant* Euonymus koopmannii. *Biodiversitas 25: 3114-3120.* Cryopreservation of endemic plants is necessary to safeguard the biodiversity and genetic resources of unique species that may face extinction due to climate change or human activities. This process enables the long-term preservation of their genetic information and ensures the possibility of restoring these species. *Euonymus koopmannii* Lauche is, known as *E. nanus* var. *turkestanicus*, is a native to Kazakhstan and other Central Asian countries. The *E. koopmannii* is listed in the Red Book of Kazakhstan and has ecological importance in soil stabilization and its decorative value. This study aims to contribute to the conservation efforts of *E. koopmannii* by establishing an effective cryopreservation protocol for its meristems. This study has identified for the first time an optimized cryopreservation protocol for *E. koopmannii* meristems involving specific treatment durations and osmotic solutions. The protocol resulted in a significantly higher survival rate (60%) when meristems were precultured with 0.3 M sucrose, treated with Plant Vitrification Solution 2 for 30 min., and regenerated on a medium with 20 g/L sucrose. Secondly, a comparative analysis of cryopreserved regenerates with a control group has revealed that cryopreservation negatively impacted the plant height but significantly increased the number of meristem shoots and roots. This finding highlights the trade-off between growth and root development in cryopreserved plants. Thirdly, the presented optimization of the cryopreservation protocol provides valuable insights for the preservation and regeneration of *E. koopmannii* meristems. It emphasizes the critical importance of precise treatments and the composition of the nutrient medium to achieve successful cryopreservation and subsequent recovery of plants in tissue culture. This research provides essential information for conserving *E. koopmannii*, shedding light on the intricate balance required to apply cryopreservation techniques effectively.

Keywords: Cryopreservation, endemic plant, *Euonymus koopmannii*, tissue culture

INTRODUCTION

Plants are paramount to humans and animals, constituting a vital component of all ecosystems. Despite their significance, the worldwide plant biodiversity tends to decrease and a continuous increase in the number of endangered and extinct species rapidly increasing yearly (Giam et al. 2010; Corlett 2016). The loss of natural populations or entire species is typically associated with destroying and altering their habitats due to the excessive human exploitation (Zhang et al. 2020; Halder and Jha 2023). Recently, environmental pollution, climate change, and infectious diseases have further exacerbated these issues, leading to a loss of genetic diversity (He and Silliman 2019; Weiskopf et al. 2020; Hohenlohe et al. 2021). *Euonymus koopmannii* Lauche (synonyms: *E. nanus* var. *koopmannii* (Lauche), *E. n.* var. *turkestanicus*) is a species of the genus *Euonymus* within the family Celastraceae. The genus *Euonymus* encompasses approximately 220 species of deciduous and evergreen shrubs, small trees, and vines (Li et al. 2021). *Euonymus* species have soil-protective significance in their habitats, as they help stabilize gullies, riverbanks, and forested slopes. Moreover, many *Euonymus* species are rightfully considered ornamental plants in urban landscaping, exhibit exceptional beauty during autumn, and are successfully localized in many countries. The *Euonymus* species are predominantly distributed in East Asia, Europe, Australia, North America, and Madagascar, with around 50 species endemic to China (Li et al. 2014).

The *E. koopmannii* is a semi-evergreen shrub up to 0.5 meters in height. It grows on the shady rocky slopes of mountains, in forests and river valleys, as well as among the bushes. It is a shrub about 2-3 m high, stems mature underground, upright shoot and often directed upward. The branches are thin, polygonal, and covered with thick leaves. The flowers are solitary or gathered in 2-3 and half umbels form a bouquet. The root bark contains gutta-percha (a hard, skin-like product). The fruits turn pink when ripe. The *E. koopmannii* is propagated by seed and vegetatively. It blooms in June and bears fruit in July and August. The fruit is a pear or heart-shaped pod with four leaves (Ivashchenko 2012). The *E. koopmannii* is found in the Ugam Range of the Talas Alatau, the Sary-Chelek Biosphere Reserve and the Aksu-Zhabagly Nature Reserve of Kazakhstan (Shukurov 2005). It is a rare relic species, protected and listed in the "Red Book" of Kazakhstan.

The conservation of endemic species is a nationwide issue that can be addressed through various methods, including seed collection of endangered species and their storage in cryobanks (Coelho et al. 2020). The preservation of ecosystems and biodiversity in their natural habitats

represents the most suitable approach for species conservation, including endemic species, as it maintains the original genetic and geographic centers of biodiversity. However, various strategies and methods should be implemented for a more comprehensive and effective conservation program, complementing and supporting in situ protection measures (Bhatia and Dahiya 2015). One of the alternative approaches to conserving endangered species is the application of biotechnological methods, including cryopreservation (Cruz-Cruz et al. 2013; Olomola et al. 2019; Halmagyi et al. 2020).

Cryopreservation involves several stages, including selecting suitable plant tissues, pre-treatment to enhance their resistance to freezing and thawing, cryoprotection to prevent ice crystal formation, rapid freezing, and long-term storage in liquid nitrogen (Bettoni et al. 2021). Cryopreserved materials can be thawed and regenerated as needed through seed germination, tissue regeneration, or other advanced methods such as somatic embryogenesis or protoplast fusion techniques (Aladele et al. 2022; Mohammadi et al. 2023; Sarma et al. 2023; Khezri et al. 2024).

Excised shoot apical meristems are the most commonly used explants for cryopreservation by vitrification. Apical meristems contain minimal infectious agents, reducing the risk of contamination and providing cleaner and more viable material for long-term storage. This factor is particularly important for the preservation of genetic material of rare and valuable plant species, as minimizing the risk of infection contributes to the success of cryopreservation. The successful application of cryopreservation by vitrification of apical meristems of rare and endangered species has been reported (Chauhan et al. 2021; Maślanka and Szewczyk 2021; Kaviani and Kulus 2022). However, it requires careful planning, research, and collaboration among conservationists, botanists, and biotechnologists to identify suitable plant materials, optimize cryopreservation protocols, and establish facilities for long-term storage.

The objective of this study is to establish an effective cryopreservation protocol for *E. koopmannii* meristems to aid in the conservation of this ecologically significant and endangered species, and to enhance survival rates and provide valuable insights into the preservation and regeneration of *E. koopmannii*. The results of this research will provide fundamental information useful for researchers in preserving the species of the genus *Euonymus*.

MATERIALS AND METHODS

Plant materials

The *E. koopmannii* experimental material was gathered from the Aksu-Jabagly Nature Reserve (42°24'25"N 70°34'49"E) in Turkistan region of Kazakhstan, under the supervision of botanists. The apical meristems from the experimental materials, grown in Murashige-Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 1 mg/L 6-Benzylaminopurine (BAP) and 0.5 mg/L Naphthaleneacetic Acid (NAA) were chosen as the initial explants for this research.

Cryopreservation solutions

Initially, there was a pre-cultivation in standard MS media with varying concentrations of sucrose (0-0.4M). Subsequently, osmoprotectants, 2M glycerol and 0.4M sucrose were used. It was followed by treatment with cryoprotectants, namely Plant Vitrification Solution 2 (PVS2), which was prepared by using a full liquid MS medium supplemented with 30% glycerol, 15% ethylene glycol, 15% Dimethyl Sulfoxide (DMSO), and 0.4M sucrose. Post-cryogenic culturing occurred in MS medium with varying concentrations of sucrose (20-30 g/L) and the addition of 0.2 mg/L kinetin. The experiments were conducted with three replicates.

Cryopreservation procedures

The experimental protocol incorporated the preculturing of meristems in MS medium supplemented with various sucrose concentrations, followed by comparative analyses to determine the most effective concentration for enhancing meristem viability during cryopreservation as adopted by Koroleva et al. (2023). Under aseptic conditions within a laminar flow hood, apical meristems of *E. koopmannii*, ranging from 0.2 to 0.4 mm, were carefully isolated from in vitro-grown young shoots using a stereomicroscope. The procedural steps undertaken were as follows:

Isolation and preculture

Meristems were first isolated and then precultured on MS medium supplemented with sucrose at 24°C for one day to prepare the tissues for cryopreservation.

Osmoprotective treatment

For 15-30 minutes, meristems were subjected to an osmotic solution containing 2M glycerol and 0.4M sucrose to optimize cell osmoregulation.

Cryoprotectant treatment

PVS2, a cryoprotectant solution, was employed for 15-40 minutes to enhance the meristem ability to withstand the freezing stress.

Controlled freezing

A cryocooler was utilized to reduce the temperature to -70°C, followed by immersing meristems in liquid nitrogen at -196°C for long-term storage.

Thawing and regrowth

After cryopreservation, the meristems underwent rapid thawing and were transferred to a regrowth medium containing various concentrations of sucrose and MS nutrients, which supported plant regeneration under specific photoperiodic conditions. A comparative study of cellular regeneration of *E. koopmannii* after cryopreservation, as opposed to control groups, over three passages (1 passage = 28-30 days) was conducted to elucidate the influence of cryopreservation on the regeneration process and the holistic restoration of the plant's anatomical structure.

Each of these steps was implemented in a precise sequence to ensure the integrity of the meristems

throughout the cryopreservation process and to promote their successful revival and subsequent growth in culture.

Statistical analysis

Morphometric data, such as root length, stem length, number of meristematic foci, number of leaves, and number of roots, were measured and presented as means with standard deviations and were analyzed using MS Excel. The mass was calculated using the formula W/V × 100%, where W indicated the weight and V indicated to volume. Statistical significance was tested using t-tests, Kruskal-Wallis test and Analysis of Variances (ANOVA). Survival rates were analyzed using the Kaplan-Meier estimator, and curve comparisons were conducted with the log-rank test using GraphPad Prism 10.1.0.316 (Mitteer et al. 2020).

RESULTS AND DISCUSSION

The optimal solutions for cryopreservation of *E. koopmannii*

The experimental data showed that survival rates of meristems were dependent on the concentration of sucrose in the preculturing medium. Meristems precultured without sucrose had a survival rate of only 20% in the control group. While, the increase in sucrose concentration to 0.2M doubled the survival rate (up to 43.3%). The highest survival rate (56.7%) was observed at 0.3M concentration, indicating an optimal osmotic environment at this concentration. However, a marginal decline in survival rate (53.3%) was recorded with a further increase in sucrose concentration (0.4M) (Figure 1). All comparisons between cantrol group and varius consentrations showed non-significant differences with p-values above 0.05.

The results of influence of osmopriming time treatment demonstrated, that the control group exhibited a minimum survival rate of 6.7%, providing a fundamental understanding of the intrinsic resilience of the meristems in the absence of any protective intervention. The survival rate increased fivefold by providing the osmopriming treatment for 15 minutes to approximately 33.3%. A further increase in treatment time to 20 minutes resulted in the peak of meristem viability, with an impressive survival rate of 60%. When the treatment time was extended to 30 minutes, a decrease in survival (46.7%) was observed (Figure 2).

There were statistically significant differences between the control group and the group treated with 15 and 30-minute time periods ($p < 0.05$). However, the differences between the control group and the group treated with a 20-minute time period were not statistically significant.

Optimal treatment duration for *E. koopmannii* meristem survival with PVS2

The control group exhibited a constant survival rate of 100% throughout the observation period, serving as a benchmark for the highest viability and demonstrating the natural longevity of meristems in an unaltered state. Treatment with PVS2 for 15 minutes resulted in an initial post-thaw survival rate of 80.16%. Although promising, the following weeks showed a steep decline in viability, reaching approximately 34% by the second week and stabilizing at 29.14% for the remaining two weeks. Extending the treatment to 20 minutes resulted in a comparable initial survival rate of 88.61%. However, a moderated decline was observed, with the rate dropping to 63.18% in the second week and further declining to 46.55% by the fourth week. Meristems exposed to PVS2 for 25 minutes maintained a consistent initial survival rate of 88.61%. Remarkably, the survival rates showed resilience, with a moderate decline to 75.95% by the second week and continuing to 65.1% by the fourth week. The 30-minute PVS2 treatment proved the most effective, achieving an initial survival rate of 93.02%. Notably, there was a minimal decline, as the survival rate remained steady at 79.73% throughout the subsequent weeks, underscoring the 30-minute duration as the superior treatment time in enhancing and maintaining meristem viability post-cryopreservation. Conversely, extending the PVS2 treatment to 40 minutes provided no additional benefits and was associated with a reduced initial survival rate of 86.49%. The subsequent decline mirrored that of the 25-minute treatment, with a final survival rate of 64.86% in the fourth week, suggesting that an overly prolonged exposure to PVS2 may not be beneficial and could potentially lead to adverse effects (Figure 3).

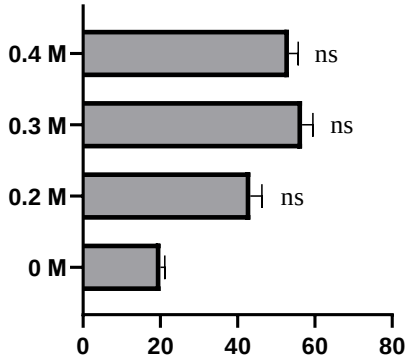

Figure 1. Survival rate (%) of apical meristems after cryopreservation at various sucrose concentrations, where ns indicate non-significant differences

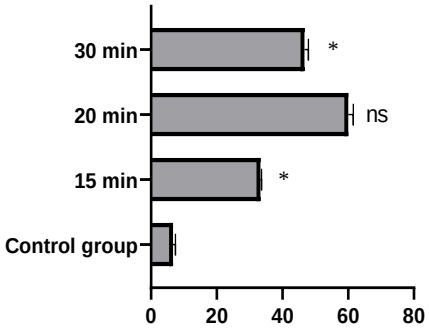

Figure 2. Influence of osmopriming time treatment (%), where ns indicate non-significant differences and *indicate significant differences at P<0.05

Optimization of nutrient medium for *E. koopmannii* meristem recovery after cryopreservation

The quantitative evaluation of meristem survival after cryopreservation was used as the primary criterion for assessing the recovery media's efficacy. The resulting data showed an apparent survival rate of 42.0% for the MS medium supplemented with 30 g/L sucrose. The MS medium with a reduced sucrose concentration of 20 g/L demonstrated superior efficacy with a survival rate of 54.7%. Contrary to the expected results, the addition of kinetin (0.2 mg/L) to the MS medium (containing 30 g/L sucrose) resulted in a marginal increase in the survival rate of 43.6% (Table 1).

Effect of cryopreservation on *E. koopmannii* cell regeneration

During the first passage, the control group exhibited an average of 3.7 leaves, while the experimental group showed a slightly lower average of 3.3 leaves. The plants in the control group had one stem, whereas the plants in the experimental group had no stems. Callus formation was observed in the plants of the experimental group, while no callus was observed in the control group. The number of roots in the control group averaged 2.3, whereas no roots appeared in the experimental group.

In the second passage, the control group had an increased number of leaves, averaging 7.3, compared to the experimental group, which showed 11.7 leaves. The control group continued to have 1 stem, while the experimental group exhibited a significant increase to 12.3 stems. Neither group showed callus formation. The number of roots in the control group averaged 4.33, whereas the experimental group still had none.

In the third passage, the control group had 13.7 leaves, while the experimental group had 14 leaves. Stems did not appear in the control group, while the experimental group showed 13 stems. Calluses were absent in both groups. The number of roots increased to 6.3 in the control group, whereas the experimental group showed a substantial increase to 21.6 roots (Table 2).

Effects of cryopreservation on *E. koopmannii* regenerants

The study compared cryopreserved regenerants with a control group not subjected to cryopreservation procedures. The data presented in Table 3 show the results of the comparative analysis for various parameters.

Within the control group, the plants showed a pronounced height of 2.24 cm, whereas the cryopreserved regenerants demonstrated a reduced height of 1.9 cm. Regarding shoot development, the control group plants showed an average of 2.25 shoots, whereas the cryopreserved regenerants presented an increased average of 10.5 shoots (Figure 4). Leaf metrics revealed that the control group plants had an average of 16 leaves, while the cryopreserved regenerants showed a slight increase with an average of 18 leaves. Regarding root characteristics, the control group plants had an average of 4 roots with a root length of 2.31 cm. In contrast, the cryopreserved regenerants revealed a significant (Table 3) increase in the number of roots, with an average of 22.5 roots, coupled with a reduction in root length to 1.4 cm.

Discussion

Genetic diversity is a critical aspect of any conservation effort, and it is important to consider the potential impact of loss of genetic diversity due to the selective preservation of specific genotypes through cryopreservation. In this study, the endangered status of *E. koopmannii* highlighted the urgency for developing efficient conservation techniques (Zhen et al. 2007; Yan et al. 2014). Our research presented an advanced cryopreservation protocol for *E. koopmannii* meristems, incorporating cryogenic science as a central conservation strategy for this endangered species, a strategy highlighted by its previous applications in the conservation of the genetic resources of various plant taxa (Gavrilenko et al. 2019; Roque-Borda et al. 2021). Optimization of cryopreservation protocols for *E. koopmannii* meristems is crucial for their successful preservation and potential future reintroduction into the wild or for use in plant breeding programs. Particular note was the survival result associated with the 0.3M sucrose concentration, noting that similar results were obtained by Le et al. (2019). This concentration significantly outperformed the other concentrations tested and the control group, confirming its role as an optimal cryoprotectant. It presumably mitigates harmful effects during the freeze-thaw cycle. In addition to acting as an osmotic regulator, it is likely to provide a protective mechanism that preserves the structural and functional integrity of the meristems.

Table 1. Nutrient media for post-cryogenic cultivation

	Sucrose concentration (g/L)	Kinetin concentration (mg/L)	Survival rate (%)
MS	30	-	42.0 ± 1.5*
MS	20	-	54.7 ± 2.4*
MS	30	0.2	43.6 ± 3.3*

Note: *P <0.05

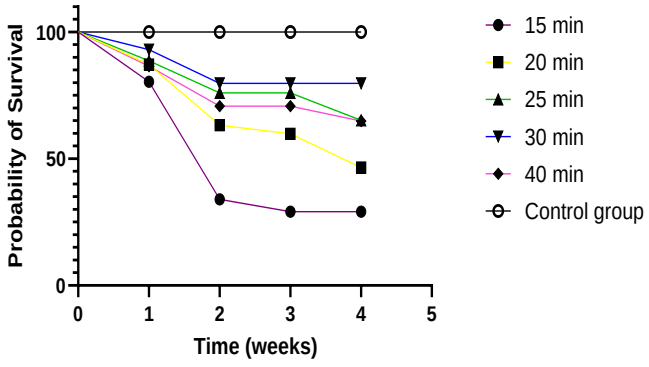

Figure 3. Survival rate (%) of meristems in cryoprotectant treatment of different time exposures for four weeks

Table 2. Morphometric parameters of plant regeneration in control and experimental groups

	Control group			Experimental group		
	First passage	**Second passage**	**Third passage**	**First passage**	**Second passage**	**Third passage**
No. of leaves	3.7±1.5	7.3±2.1	13.7±3.2	3.3±0.6 ns	11.7±4.6 ns	14.0±2.6 ns
No. of stems	1.0±0.0	1.0±0.0	1.0±0.0	0.0±0.0	12.3±5.5 ns	13.0±3.6**
No. of calluses	0.0±0.0	0.0±0.0	0.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
No. of roots	2.3±0.6	4.3±1.5	6.3±1.5	0.0±0.0**	0.0±0.0**	21.6±9.1 ns

Note: ns – not significant P>0.05;*P<0.05; **P<0.01; ***P<0.001

Table 3. The impact of cryopreservation on the regeneration

Traits	**Control group**	**Experimental group**
Length of a plants (cm)	2.2±0.5	1.9±0.7**
No. of мeristemic shoots	2.3±0.9	10.5±5.0*
No. of leaves	16.0±3.5	18.0±6.5***
No. of roots	4.0±1.0	22.5±5.8*
Length of a roots (cm)	2.3±1.3	1.4±0.2**

Note: ***P<0.001; **P<0.01; *P<0.05

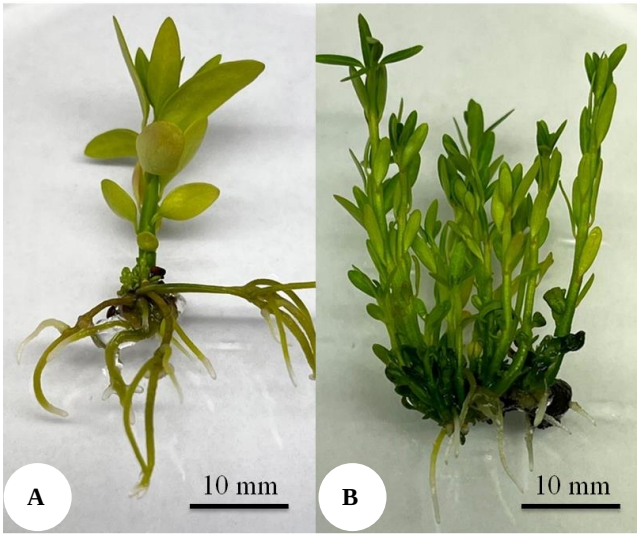

Figure 4. *Euonymus koopmannii* regeneration: A. Control group vs. B. Post-cryopreservation

Further studies may provide more insight into the underlying mechanisms by which sucrose concentrations modulate meristem viability. Sucrose has been widely used as an osmoprotectant in cryopreservation, and its ability to enhance cell receptivity and prevent damage during freezing and thawing is well-established (Zhang et al. 2015; Kisku et al. 2020). In optimizing the cryopreservation process for meristems, the temporal dynamics of exposure to osmotic solutions play a crucial role. The current study carefully evaluated the correlation between the duration of meristem treatment and subsequent survival rates after cryopreservation. The controlled treatment involved immersing the meristems in specially formulated osmotic solutions before preservation. The study also revealed the importance of the duration of the primary treatment of meristems with osmotic solutions and cryoprotectants. A treatment time of 20 minutes was optimal for the highest survival rate (60%). Longer treatment times may not significantly enhance survival, and prolonged exposure to treatment solutions may negatively affect the meristems (Normah et al. 2019).

The study's results demonstrated the effectiveness of different cryopreservation parameters on the survival and viability of *E. koopmannii* meristems. PVS2 as a cryoprotectant was successful, leading to a high survival rate of 79.73% after a 30-minute exposure. However, O'Brien et al. (2021) achieved the highest survival rate with a 20-minute treatment. PVS2 has been widely adopted in cryopreservation studies, and its successful use in this study further validates its efficacy in preserving meristematic tissue. Other researchers have also made such claims (Faltus et al. 2021; Zamecnik et al. 2021).

Optimization of the nutrient medium for the recovery of cryopreserved meristems revealed that the MS medium containing 20 g/L sucrose showed the highest percentage of survival. The increased survival rate in this medium is thought to be due to an optimal concentration of sucrose that synergizes with the metabolic requirements of the meristem. The suggestion that kinetin supplementation would improve survival rates was not supported by the survival percentage data, indicating no significant beneficial effect when added to the MS medium with 30 g/L sucrose. This finding is crucial as it provides valuable information on the most appropriate medium for the successful regeneration of cryopreserved meristems.

The regenerative capacity of cryopreserved meristems was compared to a control group that did not undergo cryopreservation procedures. The cryopreserved regenerants showed a delayed stem and root formation in the first passage. However, over several passages, complete restoration of the plant structure was observed, indicating the recovery of plant functions. Thus, the results of this comparative study underline that the regeneration of *E. koopmannii* cells after cryopreservation may encounter obstacles, resulting in the delayed or no formation of stems and roots. In contrast, in the control group without cryopreservation, regeneration proceeds without callus formation, culminating in the complete restoration of the plant structure after successive passages. These findings are of considerable importance for advancing our understanding of the intricate mechanisms underlying plant cell regeneration and developing strategic approaches for successful regeneration following cryopreservation.

These results highlight a significant increase in the number of roots in the regenerated plants following cryopreservation, albeit with a concomitant decrease in root length. Furthermore, it is noteworthy that the knowledge gained from this methodology extends beyond cryopreservation, providing valuable insights into plant propagation strategies. Thus, the optimized cryopreservation protocol of *E. koopmannii* allows for efficient preservation and recovery of meristems with minimal damage and high survival rates.

It is important to acknowledge that further research are needed to completely understand the underlying mechanisms of cryopreservation in *E. koopmannii* meristems. Further studies should explore the long-term stability of cryopreserved meristems and the potential effects of cryopreservation on the genetic integrity and phenotypic characteristics of regenerated plants. Furthermore, studies on the successful reintroduction of cryopreserved *E. koopmannii* into natural habitats or their use in plant breeding programs would be valuable for practically implementing this conservation strategy (Maschinski and Albrecht 2017). This study contributes to the growing body of research on cryopreservation as a valuable tool for conserving endangered plant species. Figure 1 provides the optimized cryopreservation protocol for *E. koopmanni* meristems.

In conclusion, this optimized cryopreservation protocol for *E. koopmannii* meristems in this study has several valuable applications including the preservation of specimens for ex-situ use, establishment and maintenance of germplasm collections, study and preservation of the genetic diversity of *E. koopmannii*, and the development of molecular markers, such as DNA barcodes, for genetic analysis of different *E. koopmannii* populations. With continued research and implementation, cryopreservation has the potential to play a significant role in preserving the biodiversity of endangered plants for future generations.

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REFERENCES

Aladele SE, Afolayan G, Jamaleddine ZO, Oduoye OT, Bolatito OA, Afolayan A, Olosunde AA, Olubiyi MR, Okere AU, Daniel IO, Ubi BE. 2022. Biotechnological approaches for genetic resources conservation and use. In: Obembe OO, Ekundayo EO, Okoli AS, Gidado A, Adetunji CO, Ibrahim AB, Ubi BE (eds). Agricultural Biotechnology, Biodiversity and Bioresources Conservation and Utilization. CRC Press, Boca Raton. DOI: 10.1201/9781003178880-24.

Bettoni JC, Bonnart R, Volk GM. 2021. Challenges in implementing plant shoot tip cryopreservation technologies. Plant Cell Tiss Organ Cult 144: 21-34. DOI: 10.1007/s11240-020-01846-x.

Bhatia S, Dahiya RS. 2015. Concepts and techniques of plant tissue culture science. In: Bhatia S, Sharma K, Dahiya R, Bera T (eds). Modern Applications of Plant Biotechnology in Pharmaceutical Sciences. Academic Press, London, United Kingdom. DOI: 10.1016/B978-0-12-802221-4.00004-2.

Chauhan R, Singh V, Keshavkant S, Quraishi A. 2021. Vitrification-based cryopreservation of in vitro-grown apical meristems of *Chlorophytum borivilianum* Sant et Fernand: A critically endangered species. Proc Natl Acad Sci India Sect B: Biol Sci 91: 471-476. DOI: 10.1007/s40011-021-01260-z.

Coelho N, Gonçalves S, Romano A. 2020. Endemic plant species conservation: Biotechnological approaches. Plants 9 (3): 345. DOI: 10.3390/plants9030345.

Corlett RT. 2016. Plant diversity in a changing world: Status, trends, and conservation needs. Plant Divers 38 (1): 10-16. DOI: 10.1016/j.pld.2016.01.001.

Cruz-Cruz CA, González-Arnao MT, Engelmann F. 2013. Biotechnology and conservation of plant biodiversity. Resources 2 (2): 73-95. DOI: 10.3390/resources2020073.

Faltus M, Bilavcik A, Zamecnik J. 2021. Vitrification ability of combined and single cryoprotective agents. Plants 10 (11): 2392. DOI: 10.3390/plants10112392.

Gavrilenko TA, Shvachko NA, Volkova NN, Ukhatova YV. 2019. A modified droplet vitrification method for cryopreservation of shoot tips from in vitro potato plants. Vavilovskij Žurnal Genetiki i Selekcii 23 (4): 422-429. DOI: 10.18699/VJ19.505. [Russian]

Giam X, Bradshaw CJA, Tan HTW, Sodhi NS. 2010. Future habitat loss and the conservation of plant biodiversity. Biol Conserv 143 (7): 1594-1602. DOI: 10.1016/j.biocon.2010.04.019.

Halder M, Jha S. 2023. The current status of population extinction and biodiversity crisis of medicinal plants. In: Jha S, Halder M (eds). Medicinal Plants: Biodiversity, Biotechnology and Conservation. Springer, Singapore. DOI: 10.1007/978-981-19-9936-9_1.

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. DOI: 10.1007/s10531-020-02032-3.

He Q, Silliman BR. 2019. Climate change, human impacts, and coastal ecosystems in the Anthropocene. Curr Biol 29 (19): R1021-R1035. DOI: 10.1016/j.cub.2019.08.042.

Hohenlohe PA, Funk WC, Rajora OP. 2021. Population genomics for wildlife conservation and management. Mol Ecol 30 (1): 62-82. DOI: 10.1111/mec.15720.

Ivashchenko AA. 2012. Reserves and National Parks of Kazakhstan. Almaty Kitap, Kazakhstan.

Kaviani B, Kulus D. 2022. Cryopreservation of endangered ornamental plants and fruit crops from tropical and subtropical regions. Biology 11 (6): 847. DOI: 10.3390/biology11060847.

Khezri M, Asghari-Zakaria R, Zare N. 2024. Plant cell and tissue culture: Propagation, improvement, and conservation of medicinal plants. In: Kumar N (eds). Biosynthesis of Natural Products in Plants. Springer, Singapore. DOI: 10.1007/978-981-97-2166-5_11.

Kisku P, Sahu S, Salma U, Sinha Ray S, Saha P, Kundu S, Ali MN. 2020. Encapsulation-dehydration based cryopreservation of *Rauwolfia* shoot tips and their regeneration potential in vitro. J Crop Weed 16 (1): 82-87. DOI: 10.22271/09746315.2020.v16.i1.1276.

Koroleva OV, Molkanova OI, Vysotskaya ON. 2023. Development of cryopreservation technique for meristems of *Syringa vulgaris* L. cultivars. Intl J Plant Biol 14 (3): 625-637. DOI: 10.3390/ijpb14030048.

Le K-C, Kim H-H, Park S-Y. 2019. Modification of the droplet-vitrification method of cryopreservation to enhance survival rates of adventitious roots of *Panax ginseng*. Hortic Environ Biotechnol 60: 501-510. DOI: 10.1007/s13580-019-00150-8.

Li Y, Dong Y, Liu Y, Yu X, Yang M, Huang Y. 2021. Comparative analyses of *Euonymus* chloroplast genomes: Genetic structure, screening for loci with suitable polymorphism, positive selection genes, and phylogenetic relationships within Celastrineae. Front Plant Sci 11: 593984. DOI: 10.3389/fpls.2020.593984.

Li Y-N, Xie L, Li J-Y, Zhang Z-X. 2014. Phylogeny of *Euonymus* L. inferred from molecular and morphological data. J Syst Evol 52 (2): 1-12. DOI: 10.1111/jse.12068.

Maschinski J, Albrecht MA. 2017. Center for plant conservation's best practice guidelines for the reintroduction of rare plants. Plant Divers 39 (6): 390-395. DOI: 10.1016/j.pld.2017.09.006.

Maślanka M, Szewczyk A. 2021. Droplet-vitrification cryopreservation of *Tulipa tarda* Stapf. apical meristems. Plant Cell Tiss Organ Cult 144: 91-95. DOI: 10.1007/s11240-020-01910-6.

Mitteer DR, Greer BD, Randall KR, Briggs AM. 2020. Further evaluation of teaching behavior technicians to input data and graph using GraphPad Prism. Behav Anal (Wash D C) 20 (2): 81-93. DOI: 10.1037/bar0000172.

Mohammadi MA, Wai MH, Rizwan HM, Qarluq AQ, Xu M, Wang L, Cheng Y, Aslam M, Zheng P, Wang X, Zhang W, Qin Y. 2023. Advances in micropropagation, somatic embryogenesis, somatic hybridizations, genetic transformation and cryopreservation for *Passiflora* improvement. Plant Methods 19: 50. DOI: 10.1186/s13007-023-01030-0.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15 (3): 473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Normah MN, Sulong N, Reed BM. 2019. Cryopreservation of shoot tips of recalcitrant and tropical species: Advances and strategies. Cryobiology 87: 1-14. DOI: 10.1016/j.cryobiol.2019.01.008.

O'Brien C, Hiti-Bandaralage JCA, Folgado R, Lahmeyer S, Hayward A, Folsom J, Mitter N. 2021. First report on cryopreservation of mature shoot tips of two avocado (*Persea americana* Mill.) rootstocks. Plant Cell Tiss Organ Cult 144: 103-113. DOI: 10.1007/s11240-020-01861-y.

Olomola D, Aguda S, Olorode E, Oyediran R, Adekunle E. 2019. The application of biotechnology in biodiversity conservation. Intl J Adv Acad Res Sci Technol Eng 5 (12): 55-63.

Roque-Borda CA, Kulus D, de Souza AV, Kaviani B, Vicente EF. 2021. Cryopreservation of agronomic plant germplasm using vitrification-based methods: An overview of selected case studies. Intl J Mol Sci 22 (11): 6157. DOI: 10.3390/ijms22116157.

Sarma MK, Sharma AA, Samantara K, Wani SH. 2023. In vitro techniques in plant breeding. In: Raina A, Wani MR, Laskar RA, Tomlekova N, Khan S (eds). Advanced Crop Improvement, Volume 1: Theory and Practice. Springer, Cham. DOI: 10.1007/978-3-031-28146-4_8.

Shukurov ED. 2005. Central Asian Transboundary Project for the Conservation of Biodiversity of the Western Tien Shan. Atlas of Biological Diversity of the Western Tien Shan Book.

Weiskopf SR, Rubenstein MA, Crozier LG et al. 2020. Climate change effects on biodiversity, ecosystems, ecosystem services, and natural resource management in the United States. Sci Total Environ 733: 137782. DOI: 10.1016/j.scitotenv.2020.137782.

Yan L, Bingling L, Qian L, Yin S, Mengxue J, Chen S, Jin X. 2014. Protective effect of reducing cryotube volume on *Lilium lancifolium* and *Euonymus fortunei* shoot tips survival after cryopreservation by vitrification. In XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014). DOI: 10.17660/ActaHortic.2015.1104.39.

Zamecnik J, Faltus M, Bilavcik A. 2021. Vitrification solutions for plant cryopreservation: Modification and properties. Plants 10 (12): 2623. DOI: 10.3390/plants10122623.

Zhang J-M, Huang B, Zhang X-N, Volk GM, Zhou Y-C, Chen X-L. 2015. Identification of a highly successful cryopreservation method (droplet-vitrification) for petunia. In Vitro Cell Dev Biol-Plant 51: 445-451. DOI: 10.1007/s11627-015-9704-y.

Zhang Y, Zhang D, Li W, Li Y, Zhang C, Guan K, Pan B. 2020. Characteristics and utilization of plant diversity and resources in Central Asia. Reg Sustain 1 (1): 1-10. DOI: 10.1016/j.regsus.2020.08.001.

Zhen W, Jianzhou G, Yan L. 2007. The adaptability of plantlets regenerated from cryopreserved shoot-Tips of *Euonymus fortunei* cv.'Yuanban' in summer. Sci Silvae Sin 43 (09): 150-154. DOI: 10.11707/j.1001-7488.20070927.