

OPTIMIZATION OF THE D-CRYOPLATE TECHNIQUE FOR CRYOPRESERVATION OF *CANNABIS SATIVA* AXILLARY BUDS

Short communication

Natalia Raquel DOLCE^{1,2,*}, Silvia Cristina SCHALLER¹

¹Instituto de Botánica del Nordeste (IBONE), UNNE-CONICET,
Sargento Cabral 2131, 3400 Corrientes, Argentina

²Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste (UNNE),
Sargento Cabral 2131, 3400 Corrientes, Argentina

Received: January 2026; Accepted: April 2026

ABSTRACT

Long-term conservation of elite *Cannabis sativa* genotypes is essential to maintain genetic fidelity in clonal propagation systems. This study aimed to evaluate and optimize the D-cryoplate technique for cryopreservation of *C. sativa* axillary buds, with particular emphasis on the role of explant dehydration before cryostorage. Axillary buds from micropropagated plants of two cultivars were encapsulated in calcium alginate on aluminum cryoplates, osmotically pretreated, dehydrated for 0–90 minutes, and immersed in liquid nitrogen. After cryostorage, the explants were rapidly rewarmed, recovered in vitro, and subsequently acclimatized under greenhouse conditions. Shoot regeneration was strongly dependent on dehydration time, with the highest regeneration rates (63–70%) observed after 60–70 minutes of dehydration. These optimal times corresponded to capsule water content of 21–28%. Short dehydration periods resulted in low regeneration due to freezing injury, while excessive dehydration significantly reduced explant viability due to desiccation stress. Shoots regenerated from cryopreserved axillary buds showed typical morphological development with growth parameters comparable to those of the noncryopreserved control. These results demonstrate that precise control of explant water content is critical for successful cryopreservation, and the D-cryoplate technique is an efficient and chemically safe strategy for the long-term preservation of elite *C. sativa* clonal germplasm.

Key words: axillary buds, dehydration, germplasm conservation, hemp, in vitro propagation

INTRODUCTION

Cannabis sativa L. is a species of global importance for its psychoactive, medicinal, and industrial applications. Its pharmacological value is primarily attributed to bioactive cannabinoids, particularly tetrahydrocannabinol (THC) and cannabidiol (CBD) (Chandra et al. 2010; Zuardi 2006), while its agronomic relevance lies in fiber and biomass production (Slade et al. 2012). The rapid expansion of the cannabis industry relies heavily on clonal prop-

agation of elite genotypes, ensuring chemical uniformity, agronomic performance, and regulatory compliance (Bansal et al. 2014). This reliance on elite clonal material underscores the need for strategies that ensure long-term genetic stability.

Micropropagation is widely used for mass production of genetically uniform plants; however, prolonged in vitro culture has been associated with genetic and epigenetic instability. Accumulation of somaclonal variation, often referred to as “Muller’s ratchet” (Muller 1932; Felsenstein 1974), may lead

*Corresponding author
e-mail: ndolce@agr.unne.edu.ar

to a gradual loss of vigor and alterations in secondary metabolite profiles, including cannabinoid content (Downey et al. 2021). Although short-term studies using molecular markers have reported limited variation, whole-genome sequencing analyses have revealed extensive intraplant genetic mosaicism in *C. sativa* (Adamek et al. 2022), suggesting that conventional in vitro maintenance may be insufficient to ensure long-term genetic fidelity.

Cryopreservation, the storage of biological materials at ultralow temperatures in liquid nitrogen (-196°C), is the most reliable strategy for long-term conservation of plant germplasm (Nagel et al. 2024). Under these conditions, cellular metabolism and division are effectively arrested, preserving genetic integrity over extended periods. In *C. sativa*, the first successful cryopreservation protocol for organized tissues was reported using droplet vitrification of shoot tips treated with PVS2 (Uchendu et al. 2019). Subsequently, vitrification-based approaches were applied to nodal explants of several commercial genotypes, yielding variable regeneration rates (26.7–66.7%) and minor alterations in some secondary metabolites (Downey et al. 2021).

Despite these advances, vitrification-based methods rely on highly concentrated cryoprotective solutions, which can cause chemical toxicity and osmotic stress, particularly in sensitive genotypes (Engelmann 2011; Nagel et al. 2024). In this context, aluminum-based cryoplate techniques such as the V-cryoplate and D-cryoplate have emerged as an efficient and practical alternative. These methods enable ultrarapid cooling and warming while minimizing mechanical damage and simplifying explant handling. Notably, the D-cryoplate technique avoids exposure to vitrification solutions by inducing dehydration through controlled airflow or silica gel. This approach has proven advantageous in several plant species sensitive to chemical cryoprotectants.

To date, the D-cryoplate technique has not been reported for *C. sativa*. Therefore, the objective of this study was to evaluate and optimize cryopreservation of *C. sativa* axillary buds using the D-cryoplate approach, with particular emphasis on determining the optimal dehydration time for post-cryogenic survival and plant regeneration.

MATERIALS AND METHODS

Plant material

Adult *C. sativa* plants cultivated under greenhouse conditions (Fig. 1A) at Brest & Brest (Corrientes, Argentina) were used as the source of primary explants. Two registered cultivars, ‘Guaraní Porá’ and ‘Pasionaria’, were selected based on their high CBD content and agronomic relevance. Both cultivars are officially registered with the National Seed Institute (INASE, Argentina, EXP 37821962 and 37776245).

Disinfection, establishment, and in vitro multiplication

Young branches were collected from greenhouse-grown plants and immediately transported to the plant tissue culture laboratory at the Instituto de Botánica del Nordeste (UNNE-CONICET). The stems were defoliated and surface sterilized under aseptic conditions in a laminar airflow cabinet.

The explants were sequentially treated with 10 vol. hydrogen peroxide ($\approx 3\%$ H_2O_2) for 30 min, followed by immersion in 70% (v/v) ethanol for 1 min, and subsequently in sodium hypochlorite solution containing 1.1% (w/v) active chlorine supplemented with Triton X-100 (0.1% v/v) for 20 min. After each step, the excess solution was carefully decanted. Finally, the explants were rinsed three times with sterile distilled water to remove residual disinfectants before in vitro establishment.

Surface-sterilized stems were sectioned into nodal segments approximately 2 cm long, each containing two nodes, and cultured individually (Fig. 1B) in glass tubes (ca. 11 mL capacity) with 3 mL of MS basal medium (Murashige & Skoog 1962). The medium was adjusted to pH 5.8, gelled with 0.75% Sigma-Aldrich Agar A-1296, and sterilized in an autoclave at 120°C for 20 min. Cultures were maintained at $27 \pm 2^{\circ}\text{C}$ under a 14-h photoperiod with photosynthetically active radiation of $350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by high-pressure sodium lamps.

Shoot regeneration and contamination rates were recorded after 30 days of culture. Nodal segments from in vitro-regenerated shoots were transferred to glass jars (ca. 150 mL) containing 30 mL of fresh MS medium to support plant development (Fig. 1C).

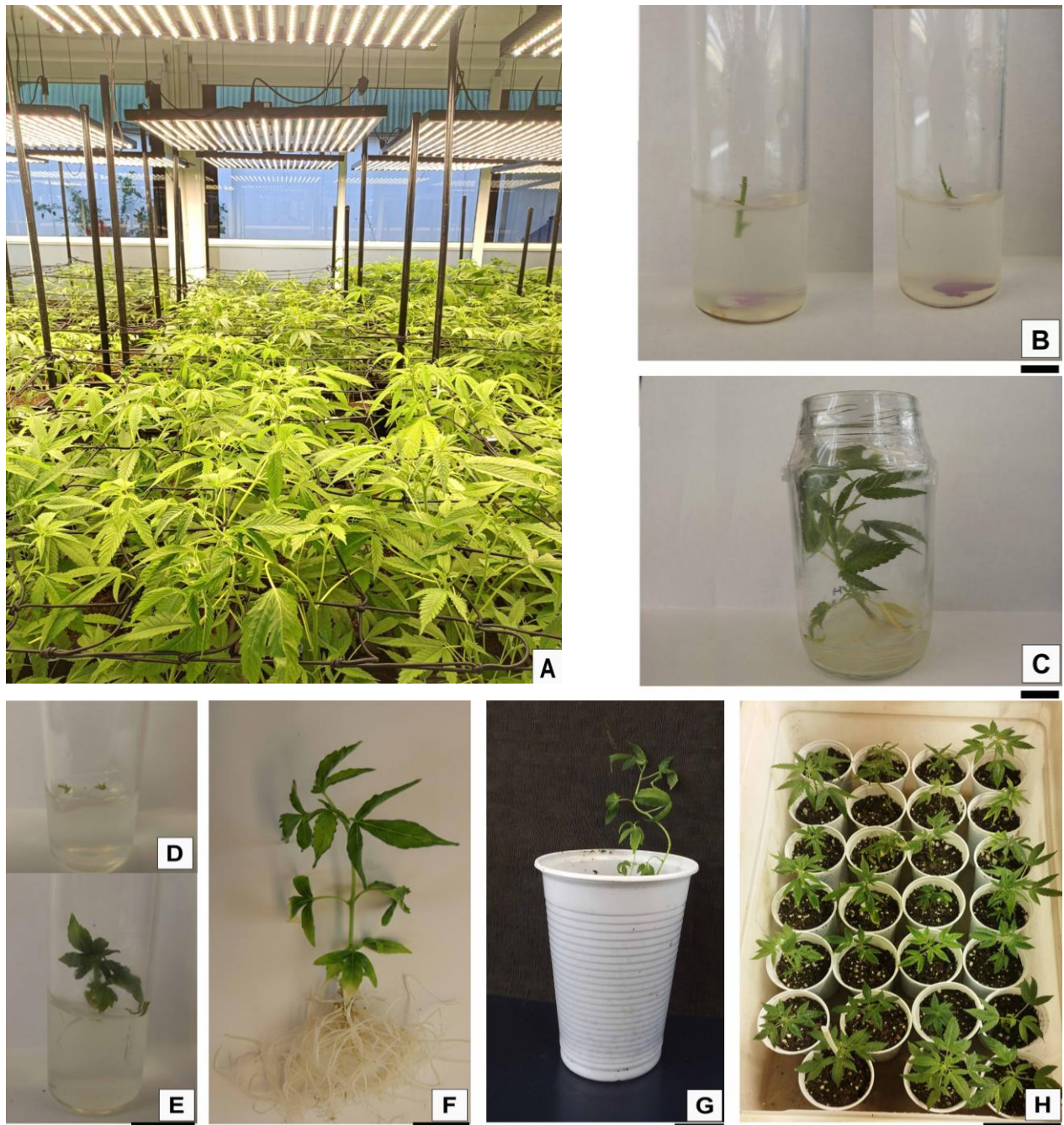


Figure 1. In vitro establishment of stem segments (B, C) of *Cannabis sativa*, excised from young branches of adult greenhouse-grown plants (A); recovery of growth and shoot regeneration from cryostored axillary buds after 5 days (D) and 30 days of culture (E); in vitro-regenerated plants after 60 days of shoot transfer to larger glass jars containing PGR-free MS medium (F, G); plants successfully transferred to soil and acclimatized to greenhouse conditions after 2 months of pot culture (H); Scale bar: B–F – 1 cm, G – 2 cm, H – 5 cm

Cryopreservation

Axillary buds (1–2 mm) excised from micropropagated *C. sativa* ‘Guaraní Porá’ plants were cryopreserved using the D-cryoplate technique described by Niino et al. (2013) with some modifications introduced in this study.

A previously reported protocol for *Ullucus tuberosus* (Arizaga et al. 2017) was used as a reference framework. The main modifications included omitting cold hardening before explant excision and using silica gel dehydration instead of air-flow dehydration.

The excised buds were precultured on semi-solid MS medium supplemented with 0.3 M sucrose for 24 h. The explants were then immobilized on aluminum cryoplates (35 × 6 mm) by encapsulation with calcium alginate. For osmoprotection, the encapsulated explants were treated with a loading solution containing 1 M sucrose and 2 M glycerol for 30 min at room temperature.

Dehydration was performed on silica gel for 0–90 min at room temperature. The cryoplates with encapsulated explants were then immersed directly in liquid nitrogen and maintained for at least 2 h.

For rewarming, cryoplates were placed directly into liquid MS medium supplemented with 1 M sucrose for 15 min. The explants were then removed from the alginate matrix and cultured on solid recovery medium.

To quantify the degree of dehydration as a function of silica gel exposure time, water content of encapsulated and cryoprotected explants was determined. The experiment consisted of 10 cryoplates per treatment, each containing 10 calcium alginate capsules with explants, and was conducted with 3 independent replicates.

At the end of each dehydration period (0–90 min), the fresh weight (FW) of the alginate capsules containing the explants was recorded using an analytical balance. The samples were then oven-dried at 75°C to constant weight to determine the dry weight (DW). Water content was expressed on a dry weight basis (%) and calculated as $[(FW - DW)/DW \times 100]$.

The optimized cryopreservation protocol developed for *C. sativa* ‘Guaraní Porá’ was subsequently applied to axillary buds of ‘Pasionaria’.

Growth recovery

Axillary buds from both cryostored and noncryostored treatments were cultured on recovery medium consisting of solidified MS medium supplemented with 0.5 mg·L⁻¹ benzylaminopurine (BAP).

Cultures were initially kept in the dark for 3 days and then transferred to standard incubation conditions for 27 days (Fig. 1D, E). After this recovery period, the regeneration rate (percentage of axillary buds that developed into shoots) and quality (stem length and node number) were evaluated.

Rooting and hardening

Shoots (~4 mm) derived from axillary buds, dehydrated for 60 or 70 min, both cryostored and noncryostored, were transferred to glass jars (150 mL) containing 30 mL of solidified MS medium to support further growth and rooting. Cultures were incubated under standard conditions for 60 days. The resulting plantlets were gently rinsed with tap water and placed in plastic pots filled with commercial soil (Growmix, Professional Multipro). The pots were kept in a humid chamber at 27 ± 2°C for 14 days. Afterward, the acclimatized potted plants were transferred to the greenhouse (Fig. 1F–H).

Statistical analysis

A completely randomized design was used for the cryopreservation experiments. Three replicates of 10 explants were performed for each treatment. After verifying normality, data were analyzed using ANOVA and Duncan’s test ($P \leq 0.05$) in InfoStat 2017 (Di Rienzo et al. 2017).

RESULTS AND DISCUSSION

In vitro establishment and multiplication

Nodal segments excised from young branches of greenhouse-grown adult plants achieved a 76% in vitro establishment rate following the disinfection protocol described above. After 30 days of culture, explants remained largely free of contamination and viable, indicating that the combined use of hydrogen peroxide, ethanol, and sodium hypochlorite provides an effective approach for surface sterilization of *C. sativa* nodal segments.

Establishing aseptic cultures from ex vitro plant material is a critical step in micropropagation systems, particularly for species such as *C. sativa*, which are susceptible to both endogenous and surface microbial contamination. As previously reported, the use of healthy stock plants combined with appropriate disinfection strategies is essential to minimize losses during culture initiation (Cassells 2001). In this study, sequential use of hydrogen peroxide, ethanol, and sodium hypochlorite resulted in aseptic explants, supporting previous findings that oxidative disinfectants can facilitate the establishment of susceptible or recalcitrant species.

Once established, nodal segments cultured on PGR-free MS medium showed efficient shoot development and multiplication, producing well-elongated shoots suitable for subsequent subculture and cryopreservation. The absence of exogenous plant growth regulators maintained normal shoot morphology and reduced the risk of physiological disorders or somaclonal variation associated with prolonged cytokinin exposure. These results confirm that PGR-free MS medium is suitable for routine in vitro propagation of *C. sativa* and provides high-quality explants for further applications, including long-term germplasm conservation.

Establishing a reliable and stable in vitro multiplication system is a prerequisite for successful cryopreservation, as the physiological status and uniformity of donor explants strongly influence post-cryopreservation survival and regeneration capacity. Therefore, the high establishment and multiplication efficiency achieved in this study provided a robust foundation for subsequent cryopreservation experiments. This observation aligns with reports emphasizing the importance of explant physiological status and uniformity for successful shoot-tip cryopreservation (Bettoni et al. 2021).

Cryopreservation and growth recovery

Figure 2 shows the effect of dehydration time on the post-cryopreservation regeneration rate of encapsulated *C. sativa* axillary buds. Shoot regeneration was strongly dependent on the degree of dehydration before immersion in liquid nitrogen. The highest regeneration rates (63–70%) were achieved after 60–70 min of dehydration, corresponding to a capsule water content of approximately 28–21%. The dehydration protocol optimized for ‘Guaraní Porá’ was subsequently applied to ‘Pasionaria’ axillary buds, which exhibited similar post-cryopreservation regeneration responses, indicating the potential applicability of the protocol across cultivars.

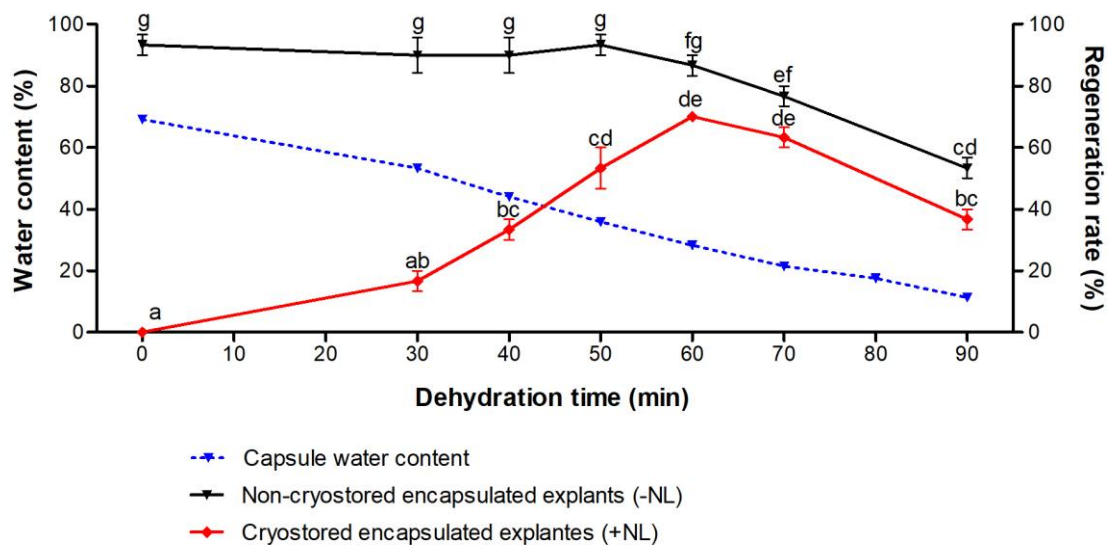


Figure 2. Effect of dehydration time on water content and post-cryopreservation regeneration rate of encapsulated axillary buds of *Cannabis sativa* ‘Guaraní Porá’ using the D-cryoplate technique compared with treated and noncryostored explants. Bars represent standard error (SE); Different letters denote significant differences by Duncan’s multiple comparison test ($P < 0.05$).

Short dehydration periods (≤ 40 min) were associated with higher water content ($>35\%$) and lower regeneration rates after cryostorage, likely due to intracellular ice formation. In contrast, prolonged (90 min) excessive dehydration, which reduced water content below 11%, significantly impaired explant viability, indicating dehydration-induced injury. These results demonstrate a narrow optimal hydration window that balances protection against freezing damage with preservation of cellular integrity.

Water content before cryostorage is a critical determinant of cryosurvival, as it determines whether cellular injury results primarily from ice crystallization or from excessive dehydration stress. In plant tissues, the “vital water” fraction, typically 20–33%, comprises nonfreezing water bound to membranes and macromolecules and is essential for maintaining the structural stability and functional protein conformation (Barraco et al. 2014; Kim & Popova 2023). The optimal water contents identified in this study fall within this physiological range, supporting the notion that the preservation of bound water is essential for successful cryopreservation.

Compared with previously reported vitrification-based protocols for *C. sativa*, the D-cryoplate technique achieved regeneration rates comparable

to or higher than these protocols while avoiding exposure to highly concentrated cryoprotective solutions. This suggests that controlled physical dehydration is a safer and more robust alternative to cryopreserving cannabis germplasm, particularly for genotypes sensitive to chemical toxicity.

In addition to regeneration rate, the quality of regenerated shoots was assessed by measuring stem length and node number (Table 1). Shoots regenerated from cryostored axillary buds exhibited growth parameters comparable to those of noncryostored explants, particularly during dehydration periods corresponding to optimal regeneration (60–70 min). These results indicate that cryopreservation using the D-cryoplate technique did not impair subsequent shoot development or morphogenetic competence. Similar observations have been reported in other species, where optimized dehydration conditions supported not only high survival but also normal post-cryogenic growth (Bettoni et al. 2021).

Overall, this study demonstrated for the first time the successful application of the D-cryoplate technique to cryopreserve axillary buds of two *C. sativa* genotypes and confirmed that precise control of explant dehydration is a key factor in maximizing post-cryogenic shoot regeneration.

Table 1. Effect of dehydration time on stem length and node number from noncryostored and cryostored axillary buds of *C. sativa* ‘Guaraní Porá’, 30 days after culture on recovery medium

Dehydration time (min)	Noncryostored		Cryostored	
	Stem length (mm)	Node number	Stem length (mm)	Node number
0	6.8 d	2.9 bcde	0.0 a	0.0 a
30	7.9 d	3.6 e	5.0 c	2.6 bcd
40	5.2 c	3.0 cde	5.0 c	2.9 bcde
50	4.5 bc	2.6 bcd	3.2 b	2.2 b
60	4.0 bc	2.7 bcd	4.5 bc	2.7 bcd
70	3.7 bc	2.5 bcd	3.8 bc	2.4 bc
90	4.1 bc	3.2 de	3.5 b	2.6 bcd

Data are presented as means of three replicates; Different letters within the same column indicate significant differences by Duncan’s multiple comparison test ($P < 0.05$)

Rooting and hardening

Shoots regenerated from axillary buds, both cryostored and noncryostored, exhibited normal growth and elongation when transferred to PGR-free MS medium. Rooting frequencies ranged from 90% to 95%, with no significant differences between treatments. The development of well-formed root systems indicates that the cryopreservation procedure did not adversely affect the rooting capacity of the regenerated shoots.

Rooted plantlets were successfully transferred to soil and acclimatized to ex vitro conditions, with approximately 90% survival after hardening. Acclimatized plants showed healthy growth, normal leaf morphology, and no visible abnormalities, indicating full physiological recovery after cryostorage. The high acclimatization survival further confirms the stability and practical applicability of the D-cryoplate cryopreservation protocol.

The ability of cryopreserved explants to regenerate whole plants and successfully acclimatize is a critical criterion for validating any cryopreservation method. The results of this study show that plants regenerated from cryostored *C. sativa* axillary buds retain normal developmental capacity, supporting the D-cryoplate technique as an effective tool for long-term conservation of elite cannabis germplasm. Similar observations have been reported for other cryopreserved vegetatively propagated species, where successful acclimatization indicates full physiological recovery (Volk et al. 2017).

CONCLUSIONS

1. A reliable in vitro micropropagation system for *C. sativa* was successfully established from greenhouse-grown plants using nodal segments cultured on PGR-free MS medium. Uniform, high-quality explants were obtained, which is an essential prerequisite for successful cryopreservation.

2. The D-cryoplate technique was used for the first time for cryopreservation of *C. sativa* axillary buds, demonstrating its effectiveness as an alternative to vitrification-based methods that rely on highly concentrated cryoprotectants.
3. Dehydration before cryostorage was identified as a critical factor determining post-cryogenic survival and regeneration, with optimal regeneration rates (63–70%) achieved at capsule water contents of 21–28%, consistent with nonfreezing water preservation.
4. Plants regenerated from cryostored explants exhibited normal growth, rooting capacity, and successful acclimatization, confirming their physiological stability and practical applicability of the optimized procedure.
5. The optimized D-cryoplate protocol described in this study is a simple, efficient, and chemically safe strategy for long-term conservation of elite *C. sativa* clonal germplasm. It provides a valuable foundation for establishing and operating cannabis cryobanks.

Author contribution statement

- NRD – A – Research concept and design, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article
- SCS – B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article

Acknowledgments

The authors thank CONICET, SGCyT-UNNE (PI 20A006), and MINCYT (SACT 759/23) for financial support. The authors are also grateful to Brest & Brest S.R.L. for providing the plant material.

REFERENCES

- Adamek K., Jones A.M.P., Torkamaneh D. 2022. Accumulation of somatic mutations leads to genetic mosaicism in cannabis. *The Plant Genome* 15(1); e20169, 13 p. DOI: 10.1002/tpg2.20169.

- Arizaga M.V., Yamamoto S.I., Tanaka D., Fukui K., No-hara N., Nishikawa T. et al. 2017. Cryopreservation of *in vitro* shoot tips of ulluco (*Ullucus tuberosus* Cal.) using D cryo-plate method. *Cryo-Letters* 38(6): 419–427.
- Bansal A., Chhabra V., Rawal R.K., Sharma S. 2014. Chemometrics: A new scenario in herbal drug standardization. *Journal of Pharmaceutical Analysis* 4(4): 223–233. DOI: 10.1016/j.jpha.2013.12.001.
- Barraco G., Sylvestre I., Collin M., Escoute J., Lartaud M., Verdeil J.L., Engelmann F. 2014. Histocytological analysis of yam (*Dioscorea alata*) shoot tips cryopreserved by encapsulation–dehydration. *Protoplasma* 251(1): 177–189. DOI: 10.1007/s00709-013-0536-5.
- Bettoni J.C., Bonnart R., Volk G.M. 2021. Challenges in implementing plant shoot tip cryopreservation technologies. *Plant Cell, Tissue and Organ Culture* 144(1): 21–34. DOI: 10.1007/s11240-020-01846-x.
- Cassells A.C. 2001. Contamination and its impact in tissue culture. *Acta Horticulturae* 560: 353–359. DOI: 10.17660/actahortic.2001.560.66.
- Chandra S., Lata H., Mehmedic Z., Khan I.A., ElSohly M.A. 2010. Assessment of cannabinoids content in micropropagated plants of *Cannabis sativa* and their comparison with conventionally propagated plants and mother plant during developmental stages of growth. *Planta Medica* 76(7): 743–50. DOI: 10.1055/s-0029-1240628.
- Di Rienzo J.A., Casanoves F., Balzarini M.G., González L., Tablada M., Robledo C.W. 2017. InfoStat versión 2017. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. <http://www.infostat.com.ar>
- Downey C.D., Golenia G., Boudko E.A., Jones A.M.P. 2021. Cryopreservation of 13 commercial *Cannabis sativa* genotypes using *in vitro* nodal explants. *Plants* 10(9): 1794; 22 p. DOI: 10.3390/plants10091794.
- Engelmann F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular and Developmental Biology – Plant* 47(1): 5–16. DOI: 10.1007/s11627-010-9327-2.
- Felsenstein J. 1974. The evolutionary advantage of recombination. *Genetics* 78(2): 737–756. DOI: 10.1093/genetics/78.2.737.
- Kim H.H., Popova E. 2023. Unifying principles of cryopreservation protocols for new plant materials based on alternative cryoprotective agents (CPAs) and a systematic approach. *CryoLetters* 44(1): 1–12. DOI: 10.54680/fr23110110112.
- Muller H.J. 1932. Some genetic aspects of sex. *American Naturalist* 66(703): 118–138. DOI: 10.1086/280418.
- Murashige T., Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15(3): 473–497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.
- Niino T., Yamamoto S.I., Fukui K., Castillo Martínez C.R., Arizaga M.V., Matsumoto T., Engelmann F. 2013. Dehydration improves cryopreservation of mat rush (*Juncus decipiens* Nakai) basal stem buds on cryo-plates. *CryoLetters* 34(6): 549–560.
- Nagel M., Pence V., Ballesteros D., Lambardi M., Popova E., Panis B. 2024. Plant cryopreservation: Principles, applications, and challenges of banking plant diversity at ultralow temperatures. *Annual Review of Plant Biology* 75: 797–824. DOI: 10.1146/annurev-arplant-070623-103551.
- Slade D., Mehmedic Z., Chandra S., ElSohly M. 2012. Is cannabis becoming more potent? In: Castle D., Murray R.M., D'Souza D.C. (Eds.), *Marijuana and Madness*, second ed. Cambridge University Press, UK, pp. 35–54. DOI: 10.1017/cbo9780511706080.
- Uchendu E., Lata H., Chandra S., Khan I.A., ElSohly M.A. 2019. Cryopreservation of shoot tips of elite cultivars of *Cannabis sativa* L. by droplet vitrification. *Medical Cannabis and Cannabinoids* 2(1): 29–34. DOI: 10.1159/000496869.
- Volk G.M., Bonnart R., Shepherd A., Yin Z., Lee R., Polek M., Krueger R. 2017. Citrus cryopreservation: viability of diverse taxa and histological observations. *Plant Cell, Tissue and Organ Culture* 128(2): 327–334. DOI: 10.1007/s11240-016-1112-4.
- Zuardi A.W. 2006. History of cannabis as a medicine: a review. *Brazilian Journal of Psychiatry* 28(2): 153–157. DOI: 10.1590/s1516-44462006000200015.