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Seed cryopreservation in *Catharanthus roseus* L., impacts on leaf structure, vegetative growth, and biochemical composition of the germinated plantlets

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Abstract: Cryopreservation offers a valuable approach for the long-term conservation of plant genetic resources, but its influence on seed viability and plant development remains underexplored. This study investigates the physiological and biochemical effects of different pre-cryopreservation drying treatments on *Catharanthus roseus* seeds. Seeds were subjected to silica gel desiccation and oven drying before immersion in liquid nitrogen (-196°C). Results showed that a moderate oven drying period (10 minutes) preserved high seed viability and led to optimal plantlet growth, mirroring untreated controls. In contrast, prolonged silica gel drying (3, and 9 hours) as well as drying for 20 min in oven significantly reduced germination rates, plantlet height, biomass, and levels of carbohydrates and alkaloids. Anatomical evaluations revealed improved vascular and mesophyll structures in plantlet leaves derived from oven-dried seeds, while moderate silica gel exposure increased epidermal thickness, indicating enhanced stress resilience. Hormonal analyses showed higher gibberellic acid (GA₃) and lower absciscic acid (ABA) levels in successfully germinated seeds. In conclusion, the study emphasizes the importance of pre-cryopreservation drying for optimizing plantlet performance and plant development with implications for pharmaceuticals. Thus, this research contributes to refining cryopreservation strategies for sustainable conservation of medicinal plants like *Catharanthus roseus* plants.

1. Introduction

Cryopreservation is a technique used to preserve plant materials by

ultra-freezing them at temperatures ranging from -80°C to -196°C. It is the most reliable method for long-term genetic resource conservation, provided that species-specific protocols are followed. The fundamental principle behind cryopreservation is to halt all cellular metabolic activities, thereby preventing cell division and significantly reducing metabolism while maintaining viability over time (Benelli, 2021). Unlike conventional freezing methods, which fail to completely arrest metabolic processes and can disrupt the distribution and concentration of essential molecules, cryopreservation effectively prevents cellular deterioration, thus ensuring successful preservation (Kaviani, 2011).

The success of plant cryopreservation largely depends on the natural ability of seeds to withstand low water content without losing viability (Walters, 2015). Reducing water content before freezing is crucial to prevent ice crystal formation within cells, which could cause structural damage (Kaviani, 2011). In general, water content reduction can be achieved in two ways: (1) using chemical cryoprotectants such as glycerol, sucrose, and dimethyl sulfoxide, which induce osmotic dehydration (Carvalho and Otoni, 2010); or (2) through desiccation, a drying method that removes water without chemical additives, minimizing potential toxicity. While desiccation is effective for seeds and pollen (Ballesteros *et al.*, 2020; Xie *et al.*, 2022), other plant materials like calli, shoot tips, and buds require chemical cryoprotectants for successful dehydration (Jenderek and Reed, 2017; Dinato *et al.*, 2020; Wang *et al.*, 2021). Achieving optimal water content is essential for vitrification, a process in which water transitions into a non-crystalline, supersaturated, amorphous solid state (Coelho *et al.*, 2017).

As seeds serve as one of the primary germplasm sources for ex situ conservation in horticulture and forestry (Li and Pritchard, 2009), establishing seed banks has become essential. The standard preservation method for most plant species involves drying seeds and storing them at -20°C (Wyse and Dickie, 2017). However, some seeds exhibit short viability and lose their ability to germinate over time (Walters *et al.*, 2005; Ballesteros and Pence, 2017; Davies *et al.*, 2018). Additionally, many seeds do not store well or fail to survive at $-18 \pm 3^\circ\text{C}$, even if they undergo desiccation (Ballesteros *et al.*, 2020). This highlights the necessity of cryopreservation for long-

term seed storage. Moreover, seed cryopreservation plays a crucial role in mitigating the effects of climate change, which can alter germination patterns. It also helps conserve endangered plant species with significant ecological and economic value (Ramanatha Rao and Parthasarathy, 2019; Dar *et al.*, 2020).

The Madagascar periwinkle (*Catharanthus roseus* L. G. Don.), a member of the Apocynaceae family, is one of the most valuable medicinal plants due to its alkaloid content. This species contains vincristine and vinblastine, two potent anticancer compounds used to treat leukemia and Hodgkin's disease (Filippini *et al.*, 2003). Given its pharmaceutical importance, *C. roseus* is highly sought after for medicinal applications. However, natural populations of this plant are limited, necessitating cultivation and conservation efforts through seed preservation. Among the available preservation techniques, cryopreservation remains the most viable method for ensuring the long-term conservation of *C. roseus* genetic resources. While cell suspension cryopreservation has been reported (Fatima *et al.*, 2009; Mujib *et al.*, 2022), no documented studies exist on seed cryopreservation for this species.

Although *Catharanthus roseus* produces orthodox seeds that can be stored successfully in conventional seed banks at low temperatures (e.g., 5°C), such storage is primarily suitable for medium-term conservation and does not fully prevent gradual physiological and biochemical deterioration over extended periods. Buchwald *et al.* (2007) showed that, despite prolonged viability at 5°C, seed aging and loss of vigour may still occur, potentially affecting subsequent plant development and metabolic performance. Moreover, conventional storage conditions do not adequately safeguard genetic integrity against long-term oxidative damage and subtle cellular alterations (Trusiak *et al.*, 2022; Tan *et al.*, 2025). Seed cryopreservation at ultra-low temperatures (-196°C) offers a distinct advantage by completely arresting metabolic activity, thereby enabling true long-term conservation with minimal risk of genetic or physiological change (Klein, 2003). In the present study, cryopreservation was therefore employed not merely as a storage method, but as a strategic conservation and experimental tool to evaluate its effects on post-germination leaf structure, vegetative growth, and biochemical composition. This approach allows assessment of

whether cryogenic exposure induces developmental or metabolic alterations compared with conventionally stored seeds, providing insights beyond seed viability alone.

Despite extensive studies on seed cryopreservation, the fundamental factors required for developing a standardized protocol remain poorly understood. Limited research has been conducted on the endogenous molecules present in zygotic embryos and germinated plantlets post-cryopreservation. Among these molecules, phytohormones play a vital role in plant growth and development (Shahid *et al.*, 2024). These plant growth regulators (PGRs) are essential for embryogenesis (Rodríguez-Sanz *et al.*, 2014; Pérez-Pérez *et al.*, 2019) and influence the growth patterns of germinated plantlets (Ghosh *et al.*, 2025). However, there is limited information regarding changes in endogenous hormone levels following seed cryopreservation.

This study aims to investigate possible alterations in two key phytohormones, gibberellic acid (GA₃) and abscisic acid (ABA), in plantlets derived from cryopreserved *C. roseus* seeds, given their crucial roles in seed germination and plant development (Abley *et al.*, 2021). The primary objective is to establish an effective, long-term seed cryopreservation protocol. We aim to assess changes in endogenous GA₃ and ABA levels in germinated plantlets. This comparison across different seed drying treatments will help identify key factors influencing the success of seed cryopreservation. In addition, carbohydrate and alkaloid content are analyzed. By addressing the knowledge gaps in seed cryopreservation, this research improves our understanding of the physiological and biochemical response of plantlets derived from cryopreserved seeds. The findings contribute to identifying how different pre-cryopreservation drying treatments influence post-germination performance in *Catharanthus roseus*, thereby supporting the optimization of seed cryopreservation protocols for conservation and pharmaceutical applications.

2. Materials and Methods

Plant materials

Catharanthus roseus L. G. Don. seeds were collected in March 2024 from plants grown in the

Ornamental Horticulture Nursery at Cairo University, Egypt. (30°01'42.6'' N, 31°12'28.4'' E). The seeds were then transported to the Laboratory of Ornamental, Medicinal, and Aromatic Plants, Faculty of Agriculture, Cairo University, for cryopreservation experiments.

Seed drying treatments and water content

The seeds were wrapped in aluminum foil envelopes 100 seeds per each and weighed to determine their wet weight. Two drying methods were applied: (1) desiccation using silica gel and (2) oven drying at 70°C. For desiccation, three opened envelopes were incubated in a 7.27 L glass desiccator with 500 g silica gel at room temperature for 3, and 9 hours. For the oven drying, the seeds were incubated at 70°C for 10 and 20 minutes. A total of five drying treatments were performed, along with a control group of fresh seeds. After each drying period, the seed weight was recorded, and the percentage of water content (WC, %) was calculated using the following equation:

$$WC (\%) = [(W_1 - W_2) / W_1] \times 100$$

Where: W1 = initial seed weight (wet weight before drying) and W2 = seed weight after drying.

Seed cryopreservation

The seeds, after desiccation, were placed in 3-mL cryogenic tubes and directly immersed in liquid nitrogen at -196°C for 24 hours. Following cryopreservation, the seeds were warmed at room temperature (25±3°C) before germination trials. Six treatments were conducted in a completely randomized design with three replicates of 150 seeds per treatment: (1) Control (unfrozen seeds), (2) Cryopreservation without drying treatment, (3) Cryopreservation after 3 hours of silica gel desiccation, (4) Cryopreservation after 9 hours of silica gel desiccation, (5) Cryopreservation after 10 minutes of oven drying, and (6) Cryopreservation after 20 minutes of oven drying.

Seed germination performance

Following cryopreservation, the seeds were sown in germination trays containing a 2:1:1 growing mixture of coco peat, perlite, and sand whose physical and chemical characteristics were analyzed in the laboratory of Soil, Water and Environment

Research Institute (SWERI), in Agricultural Research Centre (ARC), Giza, Egypt and the results are shown in Table 1. For each treatment, the 450 seeds were evenly distributed among three trays (150 seeds per tray). Each tray was subdivided into three equal quadrants, which were considered sub-replicates, resulting in nine observational units per treatment. Germination percentage and growth parameters were recorded for each quadrant. The experiment was arranged in a randomized complete block design (RCBD) with six cryopreservation treatments and three replicates (trays) per treatment, while quadrants within each tray were treated as subsamples for statistical analysis.

Table 1 - Physical and chemical characteristics of the medium used for *in vivo* germination

Characteristic	Value
PH	6.35
Electron conductivity (EC) (ds/m)	4.54
Total nitrogen (%)	0.82
Total phosphorus (%)	0.05
Total potassium (%)	0.03
C/N ratio	80
Organic carbon (%)	50.32
Bulk density (g·cm ⁻³)	0.41

Seed germination percentage and germination speed index

Germination percentage (G%) and germination speed index (GSI) were calculated following De Oliveira *et al.* (2021) as follow:

1. Germination percentage is calculated using this formula:

$$G (\%) = (N/A) \times 100$$

where: G (%) = germination percentage, N = number of germinated seeds and A = total number of sowed seeds.

2. Germination speed index (GSI) is calculated using this formula:

$$GSI = \sum \frac{G_i}{n_i}$$

where: Gi = number of germinated seeds on a given day, and ni = number of days. *Growth parameter measurement*

The plantlets were harvested 60 days after sowing, therefore, the following data parameters on

the *C. roseus* were recorded for nine randomly selected plants with three replicates: Plantlet height (cm), plantlet fresh weight (g). In addition, all leaves in each plant were sampled to measure the number of leaf pairs (n). For dry weight determination, plantlets were air-dried for 2 days to prevent immediate heat shock to the plantlet tissue followed by being oven-dried at 70°C overnight, after which their dry weights (g) were measured according to Beecher *et al.* (2013).

Anatomy and light microscopy analysis

Plantlet leaves were collected for anatomical analysis three months post-germination. The leaves were cut into small pieces and fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 3 hours, followed by post-fixation with 2% osmium tetroxide for 4 hours. Dehydration was performed using an ethanol series, and samples were embedded in epoxy resin as described by Hunter (1993). Semithin sections (500 nm) were obtained using a Leica Ultracut UCT Ultramicrotome and stained with toluidine blue. Sections were examined under a Leica optical microscope, and images were captured using the Leica Light Image Analysis System DM 750 at the Cairo University Research Park (CURP), Faculty of Agriculture.

Chemical composition analysis

Carbohydrate content. Samples (0.5 g) of dried plantlets, after removing the roots, were ground and mixed with 1 mL of 5% phenol, 5 mL concentrated sulfuric acid, and deionized water (up to 1000 mL). The mixture was heated in a water bath, followed by the addition of 12 mL NaOH to 15 mL of solution before reheating. After cooling, absorbance was recorded at 490 nm using a UV/VIS spectrophotometer (Jenway, England) against a blank (deionized water). D-glucose served as the standard, and carbohydrate content was determined as described by Dubois *et al.* (1956).

Total alkaloid content. A 100 g plantlet sample was ground and extracted overnight in methanol using a Soxhlet apparatus. The methanol extract was evaporated under vacuum at 45°C and dissolved in diluted HCl. To detect alkaloids, 2-3 drops of Dragendorff's reagent were added; a crystalline precipitate indicated the presence of alkaloids. Dragendorff's reagent with caffeine was used as the standard, and total alkaloid concentration was determined following Ajanal *et al.* (2012).

Endogenous GA_3 and ABA determination. Fresh plantlets were frozen in liquid nitrogen ($-196^{\circ}C$ for 20 seconds) and stored at $-80^{\circ}C$. Samples were ground and extracted in 80% methanol (15 mL/g FW) with butylated hydroxytoluene (BHT) as an antioxidant at $4^{\circ}C$ in darkness. The extract was centrifuged at 4000 rpm, and the supernatant was reduced to 10 mL under vacuum at $35^{\circ}C$. An equal volume of pure ethyl acetate was added, and pH was adjusted. The extract was dehydrated using anhydrous sodium sulfate, filtered, and evaporated at $35^{\circ}C$ under vacuum. The residue was redissolved in 1 mL absolute methanol. Methylation was performed following Fales and Jaouni (1973) for GA_3 and ABA determination.

Phytohormones were quantified using an ATI Unicam gas-liquid chromatograph (610 Series) with a flame ionization detector, following Vogel (1975). A coiled glass column (1.5 m x 4 mm) was used for fractionation. Phytohormone peaks were identified using exogenous hormone standards, and concentrations were calculated using Microsoft Excel.

Statistical analysis

Data were analyzed using MSTATC software (MSTAT C, 1993). A one-way ANOVA was performed, and Duncan's multiple range test (Snedecor and Cochran, 1967) was used to determine significant differences between means at $P \leq 0.05$.

3. Results

Effect of drying methods on seed water content

Desiccation treatments were performed on freshly collected seeds, and the changes in water content percentage following these treatments are presented in figure 1. Fresh seeds without desiccation exhibited the highest water content (71.32%). Desiccation on silica gel for 3 and 9 hours significantly reduced the water content to 50.93% and 48.22%, respectively. Drying in an oven for 10 and 20 minutes further decreased the water content to 40.35% and 35.36%, ranking fourth and fifth, respectively.

Evaluation of seed germination after cryopreservation and drying treatments

Following cryopreservation preceded by different drying pre-treatments, the highest germination percentage (G%) and germination speed index (GSI) were recorded in fresh, non-cryopreserved seeds

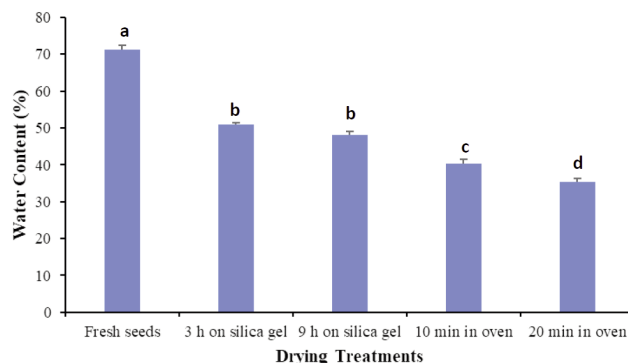


Fig. 1 - Effect of drying treatments on the water content percentage of *Catharanthus roseus* seeds. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

(control) (Fig. 2 and 3). Among cryopreserved seeds, those subjected to 10 minutes of oven drying showed the highest G% and GSI values. No significant difference in G% was observed between the control and seeds cryopreserved after 10 minutes of oven drying, as both treatments belonged to the same statistical group (Fig. 2). In contrast, a significant reduction in G% was detected in all other cryopreservation treatments, including cryopreserved seeds without any drying pre-treatment, which did not differ significantly from seeds desiccated for 3 or 9 h on silica gel or those dried for 20 minutes in the oven (Fig. 2). Regarding GSI, the control exhibited the highest value, followed by seeds cryopreserved after 10 minutes of oven drying (Fig. 3). The lowest GSI values were recorded in cryopreserved seeds without drying and in those

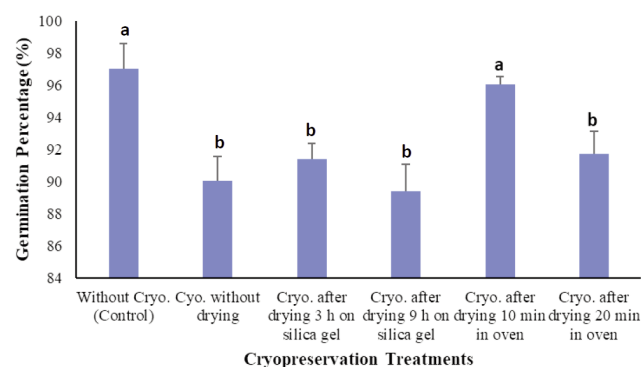


Fig. 2 - Germination percentage of cryopreserved seeds in *Catharanthus roseus* showing the effect of seed drying treatments. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

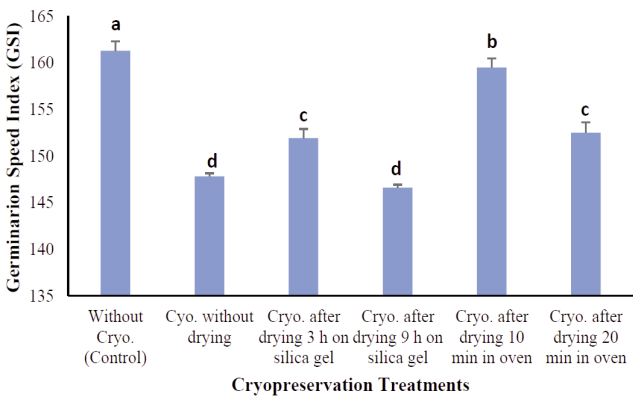


Fig. 3 - Germination speed index (GSI) of cryopreserved seeds in *Catharanthus roseus* showing the effect of seed drying treatments. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P\leq0.05$).

desiccated for 9 h on silica gel, indicating delayed germination under insufficient or excessive dehydration prior to cryopreservation. Seeds cryopreserved after 3 h of silica gel desiccation and those dried for 20 minutes in the oven showed intermediate GSI values, differing significantly from both the control and the 10-minute oven-dried treatment (Fig. 3).

Growth parameter evaluation after seed cryopreservation and drying treatments

Seed cryopreservation significantly influenced the growth parameters of *Catharanthus roseus* plantlets depending on the drying treatment applied prior to freezing (Table 2, Fig. 4). Plantlet height was significantly influenced by the drying pre-treatment applied prior to cryopreservation (Table 2). Seeds dried for 10 minutes in the oven before

cryopreservation produced plantlets with heights (13.42 cm) comparable to the non-cryopreserved control (13.54 cm), indicating that this treatment effectively preserved post-cryogenic shoot growth. In contrast, cryopreservation without drying resulted in a significant reduction in plantlet height (10.17 cm). Progressive reductions in height were observed with increasing desiccation duration on silica gel, with the 3 h treatment showing an intermediate response, while 9 h of silica gel desiccation and 20 minutes of oven drying led to the lowest plantlet heights. Overall, these results demonstrate that plantlet height is optimized within a narrow dehydration window prior to cryopreservation, and that both insufficient and excessive drying negatively affect shoot elongation. The number of leaf pairs was significantly influenced by the drying pre-treatment applied prior to cryopreservation (Table 2). Seeds dried for 10 minutes in the oven before



Fig. 4 - Development of plantlets obtained from cryopreserved seeds of *Catharanthus roseus*, after two months after sowing the seeds . (A) Without cryopreservation (control), (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven.

Table 2 - Effect of seed cryopreservation after different drying treatments on plantlet growth parameters in *Catharanthus roseus*

Seed cryopreservation and drying treatments	Plantlet height (cm)	No. leaf pairs	Fresh weight of plantlet (g)	Dry weight of plantlet (g)
Without Cryo. (Control)	13.54 ± 0.67 a	3.73 ± 0.51 a	0.65 ± 0.05 a	0.40 ± 0.02 a
Cryo. without drying	10.17 ± 0.73 b	2.60 ± 0.61 bc	0.19 ± 0.01 c	0.12 ± 0.01 bc
Cryo. after drying 3 h on silica gel	9.65 ± 0.35 bc	2.93 ± 0.72 abc	0.24 ± 0.02 c	0.15 ± 0.02 b
Cryo. after drying 9 h on silica gel	8.77 ± 0.26 c	2.73 ± 0.76 abc	0.19 ± 0.02 c	0.12 ± 0.01 bc
Cryo. after drying 10 min in oven	13.42 ± 0.48 a	3.70 ± 0.56 a	0.58 ± 0.03 b	0.39 ± 0.02 a
Cryo. after drying 20 min in oven	8.74 ± 0.48 c	3.23 ± 0.15 ab	0.23 ± 0.01 c	0.15 ± 0.01 b

Cryo.= Cryopreservation. No.= Number. Data represent the mean value ± standard deviation for three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P\leq0.05$).

cryopreservation produced plantlets with a leaf pair number (3.70) comparable to the non-cryopreserved control (3.73). Cryopreservation without drying resulted in the lowest number of leaf pairs (2.60). Seeds desiccated on silica gel for 3 or 9 h exhibited intermediate values, while 20 minutes of oven drying maintained relatively high leaf pair numbers, not significantly different from the control. These results indicate that leaf pair formation is a parameter relatively tolerant to cryopreservation stress when seeds are subjected to appropriate pre-drying. Fresh and dry weights of *Catharanthus roseus* plantlets were significantly influenced by cryopreservation and the drying pre-treatments applied prior to freezing (Table 2). The control treatment exhibited the highest fresh (0.65 g) and dry (0.40 g) weights of plantlets. Among plantlets derived from cryopreserved seeds, those originating from seeds dried in the oven for 10 minutes exhibited the highest fresh weight (0.58 g) and a dry weight (0.39 g) comparable to that of the control. In contrast, plantlets derived from cryopreserved seeds without prior drying, as well as those from seeds desiccated on silica gel for 3 or 9 h or oven-dried for 20 minutes, showed markedly reduced fresh and dry weights, with no significant differences observed among most of these treatments. These results indicate that biomass accumulation in plantlets is optimized within a narrow dehydration window prior to cryopreservation, and that fresh weight is more sensitive to cryogenic stress than dry matter accumulation. Overall, the results indicate that short oven drying (10 min) before cryopreservation preserves plantlet growth most effectively, while silica gel drying or lack of drying compromises post-cryopreservation growth performance.

Leaf anatomy of plantlets germinated from cryopreserved seeds

Anatomical analysis of *Catharanthus roseus* leaves revealed, as shown in the control (Figs. 5 A, 6A), that both upper and lower epidermal layers consisted of a single layer of cells, with stomata present on both surfaces. All examined plantlets exhibited a typical dicotyledonous leaf structure, characterized by an amphistomatic epidermis with stomata present on both adaxial and abaxial surfaces, a differentiated mesophyll consisting of palisade and spongy tissues, and a collateral vascular bundle. The upper epidermal cells were notably larger than the lower ones. The mesophyll comprised two compact layers of

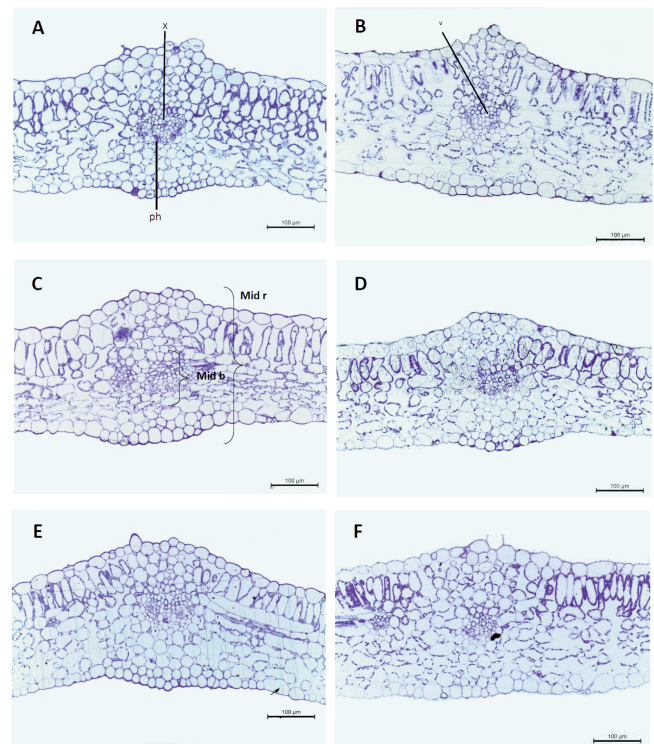


Fig. 5 - Anatomical cross sections of *Catharanthus roseus* leaf harvested from the plantlets aged two months of sowing after cryopreservation (A) Without cryopreservation (control), (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven. Mid b: midvein bundle, Mid r: midvein region, ph: phloem, v: vessel, x: xylem. Scale bars = 100 µm.

palisade cells, while the spongy mesophyll consisted of 4-5 layers of parenchyma cells with intercellular spaces and abundant chloroplasts. The vascular bundle was collateral, consisting of internal phloem and external xylem vessels.

Despite this conserved overall organization, clear treatment-dependent variations were observed in tissue thickness, cell size, and mesophyll arrangement (Table 3, Fig. 5 and 6). These variations were particularly evident in lamina thickness, palisade tissue development, spongy mesophyll compactness, epidermal thickness, and midvein bundle dimensions. The comparative ranking of treatments was based on the overall preservation of key anatomical parameters, including midvein thickness, lamina thickness, mesophyll organization (palisade and spongy tissues), epidermal thickness, and dimensions of the main midvein bundle. Control plants (without cryopreservation) exhibited the most

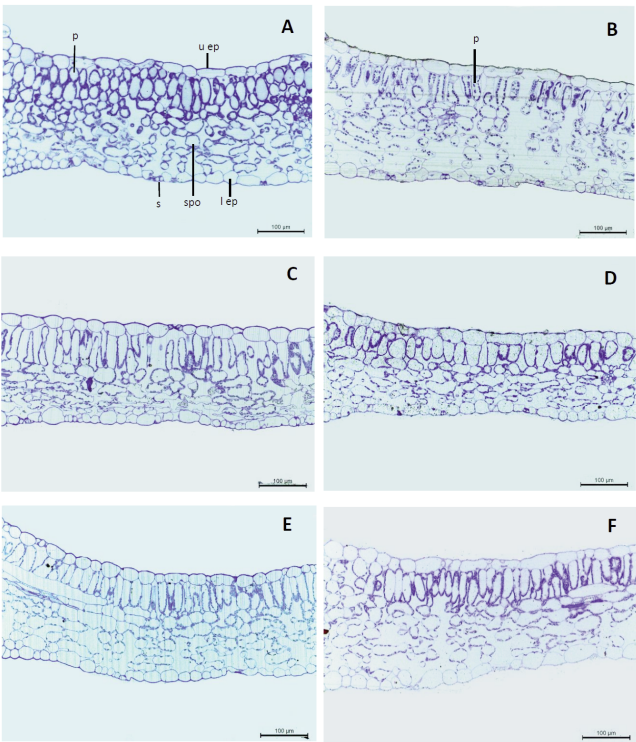


Fig. 6 - Sections of the blade leaf of *Catharanthus roseus*. The leaves are harvested from the plantlets aged two months of seeds sowing after cryopreservation showing the effect of seed drying treatments. (A) Without cryopreservation, (B) Cryopreservation without drying, (C) Cryopreservation after drying 3 h on silica gel, (D) Cryopreservation after drying 9 h on silica gel, (E) Cryopreservation after drying 10 min in oven, (F) Cryopreservation after drying 20 min in oven. l ep: lower epidermis, u ep: upper epidermis, p: palisade tissue, s: stomata, spo: spongy tissue, pal: palisade tissue. Scale bars = 100 μm.

balanced and well-developed leaf anatomy, characterized by uniform mesophyll differentiation and intact vascular tissues (Fig. 5A and 6A). Among cryopreserved treatments, plantlets regenerated from seeds subjected to oven drying for 10 min showed anatomical measurements closest to the control, including slightly higher midvein thickness (+2.08%) and main midvein bundle dimensions (+10.09%) (Table 3, Fig. 5E). This treatment also resulted in modest increases in lamina and spongy tissue thickness (Table 3, Fig. 6E), reflecting structural adjustment rather than anatomical disruption. Similarly, cryopreservation following 3 h desiccation on silica gel maintained well-organized mesophyll tissues and vascular integrity (Fig. 5C and 6C). This treatment was associated with increased thickness of the upper and lower epidermis compared with the control (Table 3), which may indicate adaptive tissue responses to dehydration stress rather than enhanced growth per se. In contrast, prolonged dehydration prior to cryopreservation, particularly drying for 9 h on silica gel, led to marked reductions in midvein thickness, lamina thickness, and palisade tissue thickness (Table 3, Fig. 5D and 6D), suggesting greater anatomical stress. The weakest anatomical development was observed in plantlets regenerated from cryopreserved seeds without prior drying, which showed reduced lamina and palisade tissue thickness and less organized mesophyll structure (Table 3, Fig. 5B and 6B). Overall, moderate drying pre-treatments before cryopreservation (10 min oven drying or 3 h silica gel drying) resulted in better

Table 3 - Measurements (μm) of some histological characters in the transverse sections of *Catharanthus roseus* leaf, showing the effect of seed cryopreservation after different drying treatments

Treatments	Histological aspects						
	Thickness of midvein	Thickness of upper epidermis	Thickness of lower epidermis	Thickness of lamina	Thickness of palisade tissue	Thickness of spongy tissue	Dimensions of main midvein bundle
Without Cryo. (Control)	281.65	18.45	16.95	155.65	71.44	84.20	80.84
Cryo. without drying	286.74	18.33	16.64	147.58	63.93	83.65	80.72
Cryo. after drying 3 h on silica gel	280.67	20.66	19.35	144.81	70.53	74.28	90.06
Cryo. after drying 9 h on silica gel	247.04	20.08	17.76	107.85	38.93	68.92	73.07
Cryo. after drying 10 min in oven	287.52	19.92	19.80	147.48	66.77	80.71	88.70
Cryo. after drying 20 min in oven	281.95	20.02	19.93	143.90	63.53	80.37	76.42

Cryo.= Cryopreservation. Means of three sections from three specimens.

preservation of leaf anatomical integrity, whereas the absence of drying or excessive dehydration adversely affected mesophyll development.

Chemical composition of plantlets after seed cryopreservation and drying treatments

Carbohydrate content. Total carbohydrate content analysis revealed that plantlets derived from cryopreserved seeds dried in the oven for 10 minutes exhibited the highest carbohydrate level (19.32%), second only to the control group (19.59%), with no significant difference between them. In contrast, the remaining treatments showed significant decreases in carbohydrate content. The lowest levels were observed in plantlets derived from cryopreserved seeds without drying, which were not significantly different from those dried for prolonged periods (3 and 9 hours on silica gel, and 20 minutes in the oven) (Table 4).

Alkaloid content. Alkaloid content followed a similar trend to carbohydrate levels. The total alkaloid content in *Catharanthus roseus* plantlets was significantly influenced by the cryopreservation and drying treatments applied to the seeds. The highest alkaloid concentration was observed in the control group (15.39 mg/100 g DW), which did not undergo cryopreservation. Among the cryopreserved groups, plantlets derived from seeds dried for 10 minutes in the oven prior to cryopreservation maintained a comparably high alkaloid content (15.21 mg/100 g DW), showing no significant difference from the control. In contrast, all other drying pre-treatments, including those without drying, dried with silica gel (3 or 9 h), and dried in the oven for 20 minutes, resulted in significantly lower alkaloid levels, ranging from 10.62 to 10.99 mg/100 g DW (Table 4).

Endogenous hormone levels (GA₃ and ABA Content). Endogenous gibberellic acid (GA₃) and

abscisic acid (ABA) levels in fresh plantlets of *Catharanthus roseus* were assessed (Fig. 7). The highest GA₃ levels were recorded in the control plantlets (11.25 µg/g FW) and plantlets derived from cryopreserved seeds following 10 minutes of oven drying (10.67 µg/g FW). Lower GA₃ levels were detected in plantlets from seeds cryopreserved after 3 hours of silica gel desiccation (7.86 µg/g FW), 20 minutes of oven drying (7.77 µg/g FW), 9 hours of silica gel desiccation (7.72 µg/g FW), and cryopreservation without drying (7.68 µg/g FW), with no significant differences among these treatments.

Conversely, ABA levels exhibited an inverse trend. The lowest ABA levels were recorded in the control (4.02 µg/g FW) and plantlets derived from

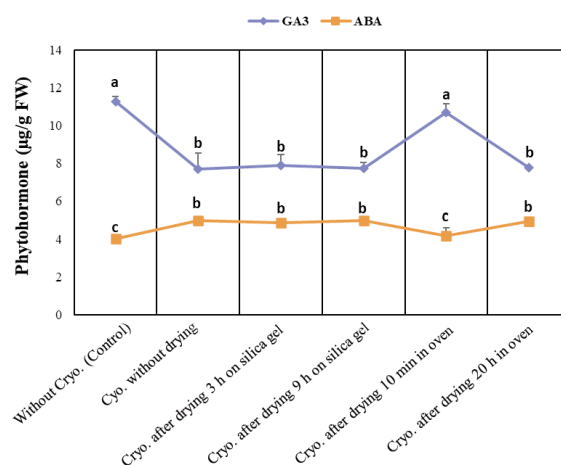


Fig. 7 - The contents of two endogenous hormones, gibberellin A3 (GA₃) and Absciscic acid (ABA) for the plantlets obtained from cryopreserved seeds in *Catharanthus roseus*. Cryo.= Cryopreservation. Error bars indicate the standard deviation of three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

Table 4 - Carbohydrate and total alkaloid contents in the plantlets development from cryopreserved seeds in *Catharanthus roseus*

Seed cryopreservation and drying treatments	Carbohydrate (%)	Total Alkaloids (mg/100 g DW)
Without Cryo. (Control)	19.59 ± 1.67 a	15.39 ± 1.11 a
Cryo. without drying	13.48 ± 1.08 b	10.62 ± 0.33 b
Cryo. after drying 3 h on silica gel	13.96 ± 1.27 b	10.78 ± 0.40 b
Cryo. after drying 9 h on silica gel	13.59 ± 0.67 b	10.67 ± 0.59 b
Cryo. after drying 10 min in oven	19.32 ± 0.68 a	15.21 ± 0.58 a
Cryo. after drying 20 min in oven	14.19 ± 0.90 b	10.99 ± 0.70 b

Cryo.= Cryopreservation. Data represent the mean value ± standard deviation for three replicates. Letters indicate significant differences for each variable according to Duncan multiple range test ($P \leq 0.05$).

cryopreserved seeds after 10 minutes of oven drying (4.19 $\mu\text{g/g}$ FW). This confirms the inverse relationship between endogenous GA₃ and ABA levels in *Catharanthus roseus* plantlets.

4. Discussion and Conclusions

This study investigated the effects of seed cryopreservation on *Catharanthus roseus*, with an emphasis on how various pre-cryopreservation desiccation treatments influence seed germination, vegetative growth, anatomical leaf development, chemical composition, and hormone levels. The findings collectively suggest that the drying method and duration prior to cryopreservation are crucial determinants of post-storage viability and performance.

Drying duration influences seed germination and plantlets vigor after cryopreservation

The study began by evaluating how different drying techniques, namely silica gel desiccation and oven drying influence seed water content. Fresh seeds had the highest water content, indicating their unaltered physiological condition. Silica gel drying reduced seed moisture progressively with time: 3 hours brought it down to 50.93%, and 9 hours to 48.22%. Oven drying, in contrast, resulted in faster moisture reduction: 10 minutes lowered the water content to 40.35%, while 20 minutes further decreased it to 35.36%. These observations support the notion that heat application accelerates desiccation but may introduce stress if overextended (Kumar *et al.*, 2023). The importance of moisture regulation prior to cryopreservation is well-documented, as excessive water can lead to intracellular ice formation, damaging cellular structures (Walters *et al.*, 2010; Campos *et al.*, 2024). Controlled dehydration prior to ultra-low temperature storage is crucial because the amount of residual water determines the likelihood of lethal intracellular ice formation during freezing, especially in intermediate seeds that do not tolerate high residual moisture (da Conceição Moreira *et al.*, 2014; Zeng *et al.*, 2024). In *Catharanthus roseus*, seeds dried on silica gel or in an oven reached lower moisture contents than fresh seeds, and the differences in desiccation rates reflect distinct dehydration mechanisms. Rapid moisture loss via oven drying (~40.35% at 10 min) likely promotes

faster removal of free water, which can help avoid ice crystallization during cryostorage (Chetverikova, 2008). However, too aggressive dehydration, as seen with extended oven drying or prolonged silica gel exposure, can itself be a stressor, compromising membrane stability and cellular function (Bharuth and Naidoo, 2020; Luro *et al.*, 2023). This duality between necessary dehydration and over-desiccation has been highlighted in studies on intermediate and partially desiccation-tolerant seeds, where moderately reduced water content enhances survival after cryopreservation, whereas either insufficient drying or excessive dehydration reduces post-thaw viability (Berjak and Pammenter, 2013). Indeed, seeds with very high moisture levels are prone to intracellular freezing injury, whereas seeds that are too dry may suffer increased oxidative stress and reduced metabolic competence for germination (Prada *et al.*, 2015). Thus, in cryopreservation protocols, balancing seed moisture content is essential to minimize both ice formation and dehydration damage, and this balance appears to be reflected in the present study's findings where moderate drying treatments resulted in better post-cryogenic performance compared with the extremes. On the other hand, too much dehydration may compromise membrane stability and enzymatic activity, reducing seed viability (Corbineau, 2024). Thus, balancing moisture content is essential.

Germination trials showed significant differences among drying treatments. Fresh seeds exhibited the highest germination percentage (G%) and germination speed index (GSI). Seeds dried in an oven for 10 minutes showed germination outcomes comparable to the control in terms of G% but with modestly reduced GSI. This suggests that brief oven drying is effective for preserving germinability while mitigating the risks of extended dehydration. These results align with studies emphasizing the need to optimize seed moisture content before cryopreservation. Ballesteros *et al.* (2021) discussed that for both orthodox and intermediate seed species, controlled dehydration to an appropriate moisture range is crucial for effective cryostorage and long-term viability, since seeds with too high or too low water content may not tolerate ultra-low temperature exposure well. Impe *et al.* (2022) also demonstrated in wheat pollen that the rate of drying and cooling interacts to influence post-cryopreservation survival, indicating that both the degree and the kinetics of dehydration are important

determinants of viability after freezing and thawing.

Seeds dried on silica gel for 3 and 9 hours showed slight declines in both G% and GSI, which may reflect marginally greater dehydration stress compared to the short oven drying. The broader literature on seed dehydration highlights physiological constraints: seeds that lose too much water may enter a state where cellular membranes are destabilized and metabolic processes are impaired, delaying germination. For example, Smolikova *et al.* (2020) reviewed mechanisms of seed desiccation tolerance, emphasizing that dehydration is a complex physiological transition involving modulation of membrane lipids and stress protective proteins; when dehydration exceeds the tolerance threshold, seed vigor and germination rates often decline. Similarly, Feng *et al.* (2017) reported that drought and dehydration stresses in plant tissues lead to altered redox balance and membrane integrity changes, which in seeds can slow or reduce germination potential, especially under severe water deficits. Although these studies focus on stress responses broadly rather than cryopreservation per se, they provide mechanistic insight into why extended dehydration may impede germination by perturbing normal physiological recovery after rehydration. Saha *et al.* (2022) further emphasize that drought-type stress responses, such as increased oxidative stress and perturbations in metabolite balance, can reduce germination performance, which is conceptually relevant here since extensive drying mimics aspects of drought stress at the cellular level. Likewise, reviews of seed deterioration processes (Choudhury and Bordolui, 2023) describe how cumulative physiological damage from prolonged water loss and associated oxidative damage contributes to reduced seed vigor and slower or incomplete germination.

Taken together, these findings suggest that moderate dehydration before cryopreservation, such as brief oven drying, can reduce free water sufficiently to minimize cryo-injury without imposing substantial physiological stress. In contrast, extended dehydration, as with prolonged silica gel exposure, may overstep the tolerance range of seed tissues, leading to subtle developmental or metabolic limitations that manifest as reduced germination performance. This highlights the need to optimize dehydration protocols to balance cryoprotective dehydration with the preservation of physiological

seed integrity.

Effects of seed cryopreservation on vegetative growth of the germinated plantlets

Vegetative growth analysis of the resulting plantlets revealed that seed cryopreservation influenced height, biomass, and leaf number. Predictably, control plants exhibited superior growth. Similar to our findings, previous work has shown that seed cryopreservation may preserve viability but alter subsequent plant development. In hybrid orchid seeds, cryopreservation did not reduce germination but led to slower long-term protocorm growth, possibly due to sublethal cellular injury caused by freezing stress (Popova *et al.*, 2003). This supports the idea that post-cryopreservation reductions in plantlet height and biomass observed in the present study may reflect physiological stress effects rather than loss of viability.

However, seeds oven-dried for 10 minutes before cryopreservation produced plantlets that closely matched the control group in height and biomass, again underscoring the efficacy of short-duration heat drying in preserving physiological integrity (Pammenter and Berjak, 2014; Zeng *et al.*, 2024). Notably, seeds cryopreserved without prior drying also showed survival and successful germination, despite having water content comparable to the control. Although vegetative growth parameters of these plantlets were generally reduced relative to the control and optimally dried treatments, their ability to regenerate suggests that *C. roseus* seeds possess a certain tolerance to cryogenic exposure even at higher moisture levels. This response may be attributed to intrinsic protective mechanisms, such as cellular compartmentalization of water or partial desiccation tolerance, which can mitigate lethal ice formation under specific conditions. However, the reduced lamina and palisade tissue thickness observed in these plantlets indicates that the absence of drying prior to cryopreservation imposed sub-lethal stress that was reflected in subsequent vegetative development. Therefore, while cryopreservation without drying did not completely inhibit survival, moderate pre-drying remains important for ensuring optimal post-cryogenic growth and anatomical integrity.

Our results suggest that oven drying may remove sufficient moisture without triggering the cellular damage seen in prolonged desiccation and drying. These results align with earlier studies indicating that

while cryopreservation may not impair seed germination per se, it can reduce growth parameters, likely due to the depletion of physiological reserves (Cejas *et al.*, 2012; Ren *et al.*, 2023). The observed variability in growth performance among the different drying treatments indicates that *Catharanthus roseus* seeds exhibit differential tolerance to pre-cryopreservation stress levels. Short-duration oven drying appears to maintain cellular and metabolic stability, whereas excessive or insufficient dehydration imposes stress that is later expressed as reduced vegetative growth. This response reflects the species-specific capacity of seeds to cope with dehydration-rehydration cycles, a trait known to vary among genotypes and to influence post-cryogenic recovery.

Anatomical Leaf structure in C. roseus plantlets after seeds cryopreservation

The anatomical analysis of leaves from different treatment groups revealed significant differences in structural traits, the overall leaf organization was conserved across all treatments, characterized by a typical amphistomatic epidermis and a dicotyledonous mesophyll composed of palisade and spongy tissues. This structural consistency indicates that cryopreservation and drying treatments did not induce fundamental alterations in leaf patterning or tissue identity. This study adds new insights, as previous anatomical evaluations focused on seed or embryo structure rather than post-cryopreservation plantlets (Schvambach *et al.*, 2022; Vargas Andriolli *et al.*, 2023).

Nevertheless, quantitative variations in tissue thickness and mesophyll organization were evident and treatment-dependent. Control plants exhibited the most balanced anatomical development, with well-differentiated mesophyll layers and intact vascular bundles. Among cryopreserved treatments, oven drying for 10 min resulted in anatomical measurements closest to the control, including increased midvein thickness and larger main vascular bundle dimensions. These changes likely represent compensatory structural adjustments that enhance vascular transport and mechanical support rather than anatomical disruption. Seeds dried on silica gel for 3 h also maintained relatively well-organized mesophyll tissues, although increased epidermal thickness was observed. Such epidermal thickening has been associated with adaptive responses to

dehydration stress, potentially enhancing protective functions without necessarily promoting enhanced growth. In contrast, prolonged desiccation (9 h silica gel drying) caused marked reductions in lamina, palisade, and midvein thickness, reflecting impaired cell expansion and mesophyll differentiation. These anatomical limitations are generally associated with reduced photosynthetic capacity and weaker vegetative growth observed in these treatments (Terashima *et al.*, 2011; Flexas *et al.*, 2012; Clarke *et al.*, 2021).

Plantlets regenerated from cryopreserved seeds without drying showed the weakest mesophyll development, particularly reduced palisade thickness and lamina depth. This supports the notion that insufficient dehydration prior to freezing permits intracellular ice formation, which, although not lethal, compromises subsequent tissue differentiation and leaf functional anatomy. These traits are linked to enhanced photosynthetic capacity and water-use efficiency (Peguero-Pina *et al.*, 2017; Agustí and Blázquez, 2020). Enhanced vascular bundle development was also observed in these groups, potentially facilitating better nutrient transport. In contrast, plantlets from undried, cryopreserved seeds exhibited the weakest anatomical characteristics, suggesting that inadequate pre-treatment compromises structural integrity (da Conceição Moreira *et al.*, 2014).

Seed cryopreservation and carbohydrate and alkaloid composition in C. roseus plantlets

Carbohydrate analysis indicated that the drying method significantly affects sugar retention. Control and 10-minute oven-dried seeds had the highest carbohydrate levels, with no significant difference between them. These findings suggest that short oven drying prevents enzymatic degradation and preserves carbohydrate reserves necessary for early growth (Pammenter and Berjak, 2014). By contrast, plantlets from cryopreserved seeds without drying, 3-, and 9- hour silica gel-dried seeds, and drying the seeds for 20 min in the oven had the lowest carbohydrate contents, likely due to prolonged stress-induced degradation. Since carbohydrates play a vital role in protecting cellular structures during freezing (Hell *et al.*, 2019; Ballesteros *et al.*, 2020), maintaining their levels is essential for post-germination success.

The study also examined alkaloid concentrations,

a key indicator of medicinal quality in *C. roseus*. Results showed that alkaloid content mirrored the carbohydrate trends. The highest levels were recorded in the control and 10-minute oven-dried samples, suggesting that these treatments effectively preserved secondary metabolites (Kumar *et al.*, 2025). Conversely, extended drying reduced alkaloid levels, likely due to oxidative degradation. These results parallel findings in cryo-derived plants where specific stressors trigger secondary metabolite accumulation, including vinblastine and vincristine, which are the essential alkaloid compounds in *C. roseus* (Mujib *et al.*, 2022; Terletskaya *et al.*, 2024).

GA₃ and ABA alterations in cryopreserved C. roseus seeds

The findings of this study highlight the effects of different drying treatments on endogenous gibberellic acid (GA₃) and abscisic acid (ABA) levels in *Catharanthus roseus* plantlets. The results indicate a significant interplay between these two phytohormones, which are known to regulate seed dormancy, germination, and plantlet development.

Gibberellic acid (GA₃) plays a crucial role in promoting seed germination, stem elongation, and other growth processes in plants (Yamaguchi, 2008). In the present study, the highest GA₃ levels were recorded in control and in plantlets from seeds cryopreserved after 10 minutes of oven drying. This suggests that short drying prior to cryopreservation maintains a hormonal balance that supports plantlet growth. Indeed, GA₃ levels were significantly lower in plantlets from seeds subjected to prolonged drying treatments. The decline in GA₃ levels with extended drying could be attributed to decrease in the plantlet growth parameters, which might impair biosynthesis or enhance degradation of this hormone (Muniandi *et al.*, 2018).

Abscisic acid (ABA) is known for its role in seed dormancy induction and stress responses (Cutler *et al.*, 2010). In contrast to GA₃, ABA levels were lowest in control and in plantlets from cryopreserved seeds after 10 minutes of oven drying. However, higher ABA level was observed in plantlets from cryopreserved seeds after silica gel desiccation, suggesting that prolonged drying before cryopreservation induces stress, leading to an upregulation of ABA biosynthesis. This aligns with previous studies demonstrating that ABA accumulation is a key response to dehydration stress

in seeds (Finkelstein, 2013).

The inverse correlation between GA₃ and ABA levels observed in this study is well-documented in plant physiology. ABA acts as an antagonist to GA₃ in regulating seed dormancy and germination, where higher ABA levels promote dormancy, while increased GA₃ levels facilitate germination and early plantlet growth (Kumar *et al.*, 2022). Our findings confirm that prolonged drying before cryopreservation disrupts this balance, leading to reduced GA₃ and increased ABA levels, which could negatively affect plantlet vigor post-germination.

The study underscores the significance of drying treatments in seed preservation and subsequent plantlet performance. While moderate oven drying (10 minutes) before cryopreservation-maintained seed viability and growth potential, prolonged desiccation negatively impacted germination, growth, and biochemical properties. These findings contribute to optimizing seed conservation protocols, ensuring enhanced viability and sustainable plant propagation strategies. The study highlights the importance of pre-cryopreservation drying techniques in preserving and even enhancing leaf anatomical features post-germination. Brief oven drying (10 minutes) before cryopreservation led to improved vascular bundle, mesophyll, and lamina thickness, while moderate silica gel desiccation (3 hours) increased epidermal thickness, potentially enhancing stress tolerance. In contrast, the weakest anatomical development was observed in plantlets from cryopreserved seeds without drying, reinforcing the necessity of proper moisture management before freezing.

In summary, seed cryopreservation in *Catharanthus roseus* induces physiological changes that influence both vegetative growth and secondary metabolite accumulation. While initial growth rates may be impacted, the overall plant vigor and metabolite production demonstrate promising adaptations. These findings contribute to our understanding of plant responses to cryopreservation and provide valuable insights for future applications in biotechnology and pharmaceutical research.

Future research should investigate how different drying treatments impact seed membrane integrity, metabolic stability, and long-term viability in cryostorage. Also, it is needed to investigate the molecular mechanisms underlying alkaloid

degradation during desiccation and cryopreservation, as well as the potential impacts on seed viability and germination success.

The results of this study highlight the potential of seed cryopreservation not only as a conservation tool but also as a means to enhance bioactive compound production in medicinal plants. The increase in alkaloid content suggests that cryopreservation could be strategically utilized in pharmaceutical industries to optimize metabolite yield. Further research should focus on optimizing cryopreservation protocols to balance plant viability with enhanced bioactive compound synthesis. Additionally, long-term field studies are needed to assess the agronomic performance and stability of cryopreserved-derived plants.

Future research should explore the physiological mechanisms behind the results obtained of anatomical analyses, such as the role of stress-related genes and proteins in maintaining cellular integrity during freezing and thawing. Additionally, further studies could investigate how these anatomical differences correlate with long-term plant health, biomass accumulation, and secondary metabolite production. Finally, localization of the studied phytohormones should be deepened to find the distribution of the endogenous hormones as a result of seed cryopreservation.

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