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Assessing different storage temperatures and protocols for preserving seeds and nodal explants of the African leafy vegetable, *Amaranthus dubius*

Ashiq Haripershad [a], Muhammad Nakhooda [b], Shakira Shaik [a,*]

[a] School of Life Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa
[b] Department of Biotechnology and Consumer Science, Cape Peninsula University of Technology, Cape Town 8000, South Africa

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ABSTRACT

This study investigated the response of seeds and nodal explants of the highly nutritious African leafy vegetable (ALV), *Amaranthus dubius*, to multiple cryopreservation protocols (controlled-rate cooling, liquid nitrogen (LN) immersion, and LN slurry (semi-solid mix of liquid and frozen nitrogen) immersion) and storage temperatures (24 (room temperature), 4 (fridge), -20 (freezer), -80 (ultrafreezer), -148 (LN vapour) and -196 °C (LN)). This study revealed that *A. dubius* seeds can be stored for 4 years at -20 and -80 °C without significantly decreasing germination capacity (86 and 88 %, respectively) compared to the non-stored control (92%). Cooling seeds at a controlled rate was found to be optimal in maintaining seed viability, irrespective of storage temperature. Controlled-rate cooling was also optimal for nodal explants across storage temperatures (-20, -80, -148, and -196 °C), which, when multiplied *in vitro*, yielded 3.6 ± 0.6 shoots per explant with an 80 % shoot development rate. To assess the effect of these treatments on genetic fidelity, a previously identified salinity tolerant *A. dubius* genotype, expressing a Na^+/H^+ exchanger (NHX1) gene, was stored, and the relative expression of the NHX1 transcript was quantified in root tissues post-acclimatisation. Nodes that were cooled at a controlled rate, in LN slurry, or in LN, maintained genetic fidelity better than those of the control, based on the relative expression of the NHX1 marker gene.

1. Introduction

The identification of preservation-tolerant genotypes within *Amaranthus dubius* populations is important in addressing the existing challenges, such as limited starting material for research applications and the gaps in current knowledge for the conservation of this nutritionally rich, versatile, and adaptable species, providing an essential resource for addressing food security, nutritional needs, and sustainable agriculture goals. Furthermore, micropropagation of superior genotypes (through direct organogenesis) allows for year-round, rapid multiplication of contaminant-free and true-to-type clones. Gaps in the current understanding of *A. dubius* responses to cryopreservation necessitate further research, specifically determining optimal preservation protocols, long-term viability and storage stability, and post-thaw regeneration. This knowledge gap is compounded by limited research on the molecular and cellular mechanistic response to cryopreservation protocols for this species.

Conventional germplasm storage protocols encompass a variety of methods aimed at preserving the genetic diversity of plant species for future use in breeding, research, and conservation efforts. These protocols typically involve the collection, preparation, and long-term storage of seeds, tissues, or whole plants under controlled conditions to maintain their viability and genetic integrity. Seeds are one of the most used germplasm storage materials due to their relative ease of collection, handling, and storage (Liu et al., 2005). As nature's germplasm conservation vehicle, they can be stored under low-temperature and low-moisture conditions to minimise metabolic activity and prolong viability. Conventional seed storage methods include drying seeds to low moisture content levels (*e.g.*, 5–10 %) and storing them in airtight containers at temperatures below freezing (*e.g.*, −18 °C or lower) (Yan 2016).

In modern plant germplasm conservation, cryopreservation has emerged as a useful approach to overcome the limitations of traditional methods of seed storage, which do not necessarily offer the same level of long-term genetic stability at minimal maintenance (Engelmann 2011). However, post-cryopreservation viability, survival, and genetic integrity of many plant species remain unknown. Cryopreservation involves preserving biological material at ultra-low temperatures, typically

* Corresponding author.
E-mail addresses: nakhoodam@cput.ac.za (M. Nakhooda), shaiksh@ukzn.ac.za (S. Shaik).

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within the liquid (−196 °C) or gaseous (~−150 °C) phase of nitrogen (Ren et al. 2021; Huang et al. 2022). Operating on the principle of vitrification, solidifying liquids without crystallising, thereby minimising harmful ice formation (Pegg 2014), cryostorage is a relatively cost-effective (Panis et al. 2020) and space-efficient method for preserving the genetic integrity, metabolic stability, and field performance of some clonally propagated crops (Vendrame et al. 2014; Niino and Arizaga 2015; Coelho et al. 2020; Bi et al. 2021; Vollmer et al. 2022). Various cryopreservation techniques have been successfully reported across diverse plants, including *Carica papaya* (Kaity et al. 2008), *Phaseolus vulgaris* (Cejas et al. 2013), *Amaranthus hybridus* (Adu-Gyamfi et al. 2014), *Malus* spp. (Wang et al. 2018), *Lilium* spp. (Li et al. 2019), *Stevia rebaudiana* (Benelli et al. 2021), *Vitis* spp. (Bettoni et al. 2021), *Olea europaea* (Bradaï and Sánchez-Romero 2021), and *Musa* spp. (Roostika et al. 2022), showcasing the applicability and relevance of cryopreservation across varying plant tissues and genotypes.

Despite its many demonstrated advantages, the cryopreservation process inherently imposes stress on plant cells, potentially inducing (*epi*)genetic modifications in cryopreserved explants and regenerated plants, leading to reduced survival and regrowth, even under optimised conditions (Cejas et al. 2013; Popova et al. 2023). The molecular stability of cryopreserved plant material remains inadequately studied, highlighting gaps in our understanding of the genetic and epigenetic changes potentially occurring during cryopreservation (Bi et al. 2021; Wang et al. 2021). The limitations and concerns linked with the plethora of cryopreservation protocols underscore the necessity for further investigation, specifically to address stresses and variations induced among superior and threatened plant genotypes and species (Lee et al. 2021; Pence and Bruns 2022). Therefore, this study aimed to investigate the growth and physiology of *A. dubius* in response to cryopreservation stresses and to evaluate the feasibility of multiple cold storage techniques for safeguarding seeds (−20 and −80 °C storage, pre-storage controlled-rate cooling, and LN slurry immersion) and nodal explants (−20 and −80 °C storage, controlled-rate cooling, LN liquid immersion, and LN slurry immersion) of *A. dubius*. This optimisation-focused approach addresses a research gap in current seed conservation practices for orphan crops and supports more robust long-term genetic resource management.

If the intention of cryopreservation is to conserve superior selected genotypes, then alternative explants (such as nodal explants) must be sought, as, given inherent variability, whole seeds are not suitable for this purpose. A viable alternative is the cryopreservation of nodal explants of superior selected genotypes, but it also requires stringent, sample-specific optimisation, often utilising cytotoxic cryoprotectants and demonstrating varying degrees of success across species and even among genotypes (Engelmann 2014; Paula et al. 2018). Hence, this method can be technically demanding to initiate, and cryoprotectant toxicity may pose a risk to cell viability (Pegg 2014; Coste et al. 2015). Nevertheless, numerous studies have reported successful regeneration of plantlets from vitrified cells, demonstrating normal development post-storage in liquid nitrogen (LN) (Prada et al. 2015; Downey et al. 2021). To further enhance survival and post-thaw characteristics, controlled-rate cooling, representing another iteration of cryopreservation, involves precisely regulating the cooling rate in programmable freezers or cooling rate-controlled freezing containers to minimise ice crystal nucleation during freezing (Kilbride et al. 2019; Wilms et al. 2020; Chang and Zhao 2021). However, implementing controlled-rate cooling systems is more complex and costly than other, simpler preservation methods like direct LN immersion (Woods et al. 2007).

After preserving nodal explants, examining the effect on the genetic fidelity of recovered genotypes is necessary. Genetic markers, including simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), amplified fragment length polymorphisms (AFLPs), and random amplified polymorphic DNA (RAPD), offer higher reliability in determining genetic fidelity compared to morphological and biochemical markers due to the direct assessment of DNA, which remains largely unaffected by environmental factors or developmental stages (Semagn et al. 2006; Mondini et al. 2009). Genetic techniques also offer higher precision than morphological and biochemical methods, which may not provide enough variability for high-resolution differentiation (Powell et al. 1996; Varshney et al. 2005). However, the above-mentioned genetic approaches lack specificity, especially pertaining to biological functionality, because only general variations are evaluated, which may not correlate with the expression of specific genes of interest (GOI) and the activity of desirable traits, such as high yield, nutritional content, and stress tolerance (Wang et al. 2021; Park et al. 2025).

To address this limitation and gain further insights into gene-specific functionality, the present study aimed to investigate the regulation of a salinity-responsive NHX1-like gene discovered in a previously identified salinity-tolerant genotype of *Amaranthus dubius* (Haripershad et al. 2024). Salinity tolerance was explored due to its critical importance in agriculture, as soil salinisation severely limits crop productivity, especially in arid and semi-arid regions where salinity stress is a significant challenge to sustainable food production (Qadir et al. 2014). Assessing the integrity and functionality of this gene, which is an important determinant of salinity tolerance in this species, post-cryopreservation is necessary for preserving the original (superior) genotype. Moreover, elucidating gene expression fluctuations in response to different storage protocols can contribute to the optimisation of cryopreservation techniques, potentially ensuring minimal disruption to molecular pathways. These analyses provide a foundation for understanding the molecular basis of stress tolerance, identifying stress-responsive genes, and developing strategies for enhancing stress resilience within the species of interest.

2. Materials and methods

The experimental design for this study is summarised in Fig. 1, which outlines the sequence of growth measurements and gene expression analyses used to screen and select preservation routes based on germination, growth, and genetic fidelity of stored seeds and nodal explants. The first step assessed germination and seedling growth parameters of multiple seed preservation protocols in a greenhouse. Then, cold storage of nodal explants was evaluated *in vitro* following greenhouse acclimatisation. Finally, the genetic fidelity of stored explants was assessed by comparing stress-responsive gene expression patterns among plantlets

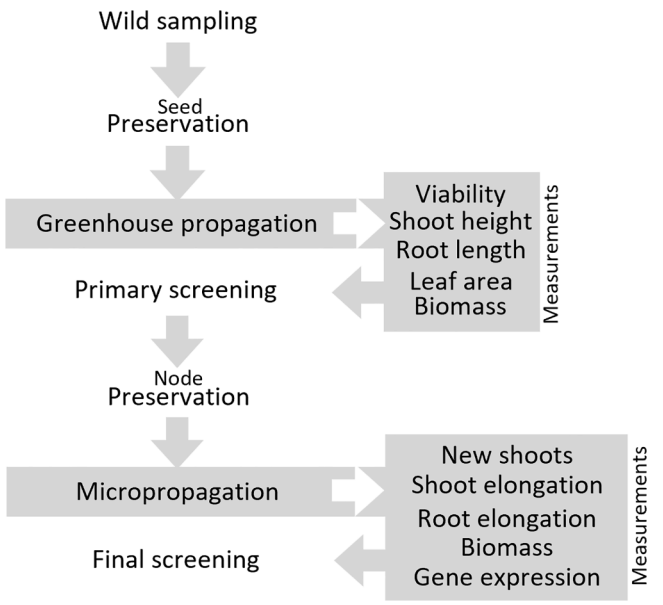

Fig. 1. Flow diagram of the experimental design for this study to quantify storage stress.

derived from a previously identified, salinity stress-tolerant *A. dubius* genotype exposed to the various freezing methods and storage conditions to a non-preserved, non-stored control.

2.1. Wild-type seed preservation

Mature, sun-dried *Amaranthus dubius* seeds with water content less than 0.01 g H_2O/g (measured after sun-drying) were harvested during September 2018 from naturally-growing populations in Desainagar, South Africa (29°36′47.1″S 31°09′08.8″E), and were hand de-chaffed, wrapped in paper towel, and then stored in an airtight glass container with silica crystals at 24 °C in the dark until experimental use, less than one month postharvest. For verification, a specimen was deposited to the Bews Herbarium in Pietermaritzburg, KwaZulu-Natal (accession: NU0094621). The seeds were surface sterilised in 70 % (v/v) ethanol (EtOH) (Protea Lab Services, South Africa) for 5 min prior to different cooling treatments and deep freeze storage. An untreated seed lot was wrapped in sterilised paper towel and stored in an airtight glass canister with silica crystals at 24 °C in the dark for 4 years ($n = 64$).

2.1.1. Nitrogen slurry cooling

Seeds were transferred to 2 mL round bottom cryo vials (Thermo Fisher Scientific, USA) and directly immersed in LN (Afrox, South Africa) slurry under vacuum, resulting in a cooling rate of more than 500 °C/s (Echlin 2013). The seeds were then stored at −20 and −80 °C (NuAire, USA) for 4 years ($n = 64$).

2.1.2. Mr Frosty® cooling

Seeds were transferred to 2 mL round bottom cryo vials and inserted into a Mr Frosty® cooling rate-controlled freezing container (Nalgene, South Africa) containing isopropanol (IPA) (Protea Lab Services, South Africa). The seeds were cooled at a rate of 1 °C/min to −40 °C by placing Mr Frosty® into a −80 °C ultra-freezer (NuAire, USA) for 4 h. The seeds were then stored at −20 and −80 °C (NuAire, USA) for 4 years ($n = 64$).

2.1.3. Freezer cooling

Seeds were transferred to 2 mL round bottom cryo vials and stored at −20 and −80 °C (NuAire, USA) for 4 years ($n = 64$).

2.2. Thawing and seed germination

After the stipulated freezing routes, the seeds were ambiently warmed at 35 °C for 3 min following removal from storage after 4 years (February 2019 to February 2023). Individual seeds were then sown into seedling trays (37 mm width x 60 mm height) containing a 1:1 mixture of sterilised coco peat and seedling mix (Grovida, South Africa). The trays were placed inside growth rooms at the School of Life Sciences (University of KwaZulu-Natal, Westville campus) and monitored daily for emergence. An untreated control was established using non-stored seeds by sowing one seed lot at the beginning ($n = 64$) of the 4-year storage period. After approximately three weeks, seedlings that developed a second set of true leaves were transplanted into plastic flowerpots (90 mm diameter x 100 mm depth) with the same substrate as the seedling trays. However, the pots were additionally supplemented with 10 % v/v vermiculite (Grovida, South Africa) to improve aeration and water and nutrient retention and exchange (Rajkumar et al. 2017; Xu et al. 2021). Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to the substrate once every seven days at a volume of 10 mL per seedling. The germination rates of preserved and untreated seeds were recorded. Furthermore, seedling height and the number of leaves and nodes were recorded before *in vitro* propagation.

2.3. In vitro propagation

Nodal explants were excised from seedlings grown from germinated cryopreserved seeds and transferred to semi-solid shoot multiplication medium (half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L indole-3-acetic acid (IAA), pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks (Shaik et al. 2022). All media were steam sterilised in an HL-341 autoclave (Gemmy Industrial Corporation, Taiwan) at 121 °C and 15 psi for 20 min. Thereafter, nodes were aseptically excised and cryoprotected with chilled 50 % Plant Vitrification Solution 2 (PVS2) (15 % glycol (Sigma, UK), 15 % dimethyl sulfoxide (Sigma, UK), 0.4 M sucrose in full-strength liquid MS) for 5 min followed by 100 % PVS2 for 15 min prior to cooling (Varghese et al., 2009). Nodal explants not preserved or exposed to seed storage treatments were used as a control.

2.4. Nodal explant preservation

Nodal explants had a water content of less than 0.01 g H_2O/g fresh weight.

2.4.1. Nitrogen slurry cooling

Cryoprotected nodal explants were directly submerged in LN slurry, as done for seeds in Section 2.1.1, and then stored at −20, −80, −148 (LN vapour), and −196 °C (LN) for 1 week ($n = 135$).

2.4.2. Liquid nitrogen cooling

Cryoprotected nodal explants were transferred to 2 mL round bottom cryo vials, inserted into CryoCanes, and plunged into LN for 2 min, resulting in a cooling rate of ~200 °C/s (Sakai 1965). The cryo vials were then stored at −20 and −80 °C for 1 week each ($n = 135$). Additionally, some CryoCanes were stored in LN vapour (−148 °C) and LN (−196 °C) for 1 week each ($n = 135$).

2.4.3. Mr Frosty® cooling

Cryoprotected nodal explants were cooled using Mr Frosty®, as done for seeds in Section 2.1.2, and stored at −20, −80, −148 (LN vapour), and −196 °C (LN) for 1 week ($n = 135$).

2.4.4. Freezer cooling

Similar to seeds in Section 2.1.3, cryoprotected nodal explants were stored at −20 and −80 °C for 1 week ($n = 135$).

2.4.5. Salinity-tolerant genotype cryopreservation

Nodal explants harvested from a previously identified salinity-tolerant *A. dubius* genotype (S4) (Haripershad et al. 2024) were also subjected to cryopreservation protocols (2.4.1 - 2.4.4). Additionally, untreated nodal explants from S4 were micropropagated via direct organogenesis (Shaik et al. 2022) and served as a control.

2.5. Thawing and recovery of nodal explants

Upon retrieval from cryostorage, cryo vials were rapidly warmed in a water bath at 40 °C for 2 min, then surface sterilised with 70 % EtOH and transferred to a laminar flow hood, wherein the PVS2 solution was replaced with 1.2 M sucrose and incubated at 24 °C for 20 min. The nodal explants were then rinsed with distilled water and recovered in sterile plastic Petri dishes containing full-strength MS liquid medium supplemented with 30 g/L sucrose at 24 °C for 30 min. Thereafter, the samples were transferred to semi-solid shoot multiplication medium (half-strength MS, 30 g/L sucrose, 2 mg/L BAP, 0.5 mg/L IAA, pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks, and finally transferred to rooting medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L IAA, pH 5.7) for a further two weeks (Shaik et al. 2022). The resulting rooted clones were acclimatised in plastic flowerpots (90 mm diameter x 100 mm depth) containing a 1:1 mixture of coco peat and seedling mix (Grovida, South Africa) inside

greenhouse mist tents and watered daily at 4 pm with municipal water for 5 min. Before the explants were subcultured from the multiplication medium to the rooting medium, the number of shoots, shoot length, and any callus formation were recorded. The number of root-producing explants, root number, and root length were recorded following the rooting phase. During acclimatisation, explant height and leaf number were recorded.

2.6. RNA extraction, quantification, and qualitative control

Following acclimatisation, salinity stress (400 mM NaCl) was incrementally applied (50 mM NaCl/day) to a subset of each S4 cryostorage group for 1 week, and then root tip tissue was randomly excised ($n = 9$). The tissue samples were rinsed with distilled water for 10 s and blotted dry with autoclaved paper towel. The samples were sectioned, weighed, and frozen in LN (Afrox, South Africa) and then immediately prepared for RNA extraction or stored at −80 °C in an ultra-freezer (NuAire, USA) until use. Total RNA was isolated from root tissue for each treatment using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. The RNA concentration was quantified using the RiboGreen® protocol for the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, USA). RNA quality was evaluated by running 5 μg of the extract through electrophoresis on 1 % Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) agarose gel (Thermo Fisher Scientific, USA) supplemented with 1 % NaOCl (Reckitt Benckiser, South Africa) for 30 min at 110 V (Aranda et al., 2012). All RNA samples were aliquoted and stored at −80 °C in an ultra-freezer (NuAire, USA) until needed for RT-qPCR analysis.

2.7. RT-qPCR parameters

RT-qPCR analysis was completed using the GoTaq® 2-Step RT-qPCR System (Promega, USA). The cDNA template was synthesised from 1 μg of total RNA per the manufacturer's instructions. The qPCR final reaction volume of 20 μl was composed of 1 μl cDNA template (equivalent to 1 ng starting quantity of RNA), 10 μl of GoTaq® qPCR Master Mix (2X), 1 μl of both primers (500 nM) (Haripershad et al. 2024), and 7 μl of nuclease-free water. No template controls (NTCs) were performed with 8 μl of nuclease-free water to account for the lack of cDNA, thus ensuring a constant final reaction volume. The RT-qPCR thermocycling reactions were performed with three biological and two technical replicates of each treatment using the Eco™ Real-Time PCR System (Illumina, USA). All assays were carried out under the following conditions: one cycle of 95 °C for two min, 40 cycles of 95 °C for 15 s and 68 °C for one min for the degenerate NHX1 primer pair and 60 °C for one min for the reference gene primer pairs. A melting curve was generated from 95 °C to 65 °C to verify amplicon specificity and identify erroneous hairpin and primer dimer formation. The size of the amplicon was estimated using gel electrophoresis (Armstrong and Schulz 2015).

2.8. RT-qPCR analysis

LinRegPCR (Ruijter et al. 2009) was used to determine the fluorescence threshold and the mean RT-qPCR efficiency per amplicon. The generated quantitation cycle (Cq) values and RT-qPCR efficiencies (E) of the NHX1 GOI and reference gene, malate dehydrogenase (MDH), were used to calculate the relative quantification (RQ) for each salt stress treatment as described by ***Equation 1*** (Pfaffl et al. 2004). These data were then expressed as relative fold changes per salt stress treatment. BestKeeper was used to assess the stability of the reference gene by calculating the standard deviation (SD) and coefficient of variation (CV %) (Pfaffl et al. 2004).

Equation 1. Pfaffl method of relative quantification:

$$RQ = \frac{(E_{unknown})^{\Delta Cq_{unknown}}}{(E_{control})^{\Delta Cq_{control}}}$$

Where: $\Delta Cq_{unknown} = Cq$ of NHX1 in control treatment – Cq of NHX1 in unknown treatment, and: $\Delta Cq_{control} = Cq$ of MDH in control treatment – Cq of MDH in unknown treatment.

The relative quantification (RQ) of the GOI was calculated by determining the RT-qPCR efficiencies (E) and differences in quantitation cycles (ΔCq) between test samples (100, 200 and 400 mM NaCl) and the control (0 mM NaCl).

2.9. Data analyses

Germination and survival rates (Chi-square test) and growth data (ANOVA) were analysed using the Statistical Package for the Social Sciences (SPSS®) version 29.0 (IBM, USA). The growth data were assessed for normality (Shapiro-Wilk test) and metavariance (Levene's test) to satisfy the assumptions of the post hoc test. Significant interactions among and between treatments were identified by univariate ANOVAs followed by Tukey post hoc testing. In instances where the assumption of homogeneity of variances was violated, the pairwise Games-Howell post hoc test was applied. The Kruskal-Wallis test was used to compare non-parametric data. A probability of $p < 0.05$ was used as the threshold for statistical significance.

3. Results and discussion

3.1. Seed cryopreservation

The 1-week germination of *A. dubius* seeds is photographed in Fig. 2 and plotted in Fig. 3, showing the results of various seed cooling and storage methods. No statistically significant differences existed between the control and all seeds stored at −20 and −80 °C for 4 years. However, there were significant differences between the seeds stored at 24, 4, and −20 °C and the control and LN slurry and Mr Frosty® cooled seed lots stored at −20 and −80 °C. The largest statistically significant difference in germination % occurred in the samples rapidly frozen in LN slurry and stored at −80 °C, yielding 94 % germination compared to untreated seeds stored at 24 °C, resulting in 82 % germination. Additionally, the observed lack of statistically significant differences between the control (92 %) and all samples stored at −20 °C (90 %) and −80 °C (90 %) for 4 years indicated the effectiveness of these storage temperatures in preserving *A. dubius* seed viability for 4 years, irrespective of the cooling method. This resilience of seeds against deterioration at ultra-low temperatures suggests that storage at both −20 °C and −80 °C are suitable conditions for this species, precluding degradation, thereby maintaining seed quality comparable to unaged seeds. This is likely due to the high surface area-to-volume ratio of the minuscule seeds in conjunction with their relatively low (< 15 %) water content (Omondi 2017).

Another *A. dubius* seed storage study conducted by Reddy (2022) assessed multiple cryoprotectants, cooling rates, and germination conditions, finding that LN immersion resulted in the highest germination (> 80 % after 24 h), in agreement with the present findings. This result is further supported by a study conducted on another amaranth, *A. cruentus,* that also resulted in better germination ability under cold storage (> 85 % germination after 300 days) (Martins et al. 2019). The effectiveness of sub-zero storage temperatures in preserving seed viability is further supported by a long-term assessment of 37 species of *Brassicaceae* (Pérez-García et al. 2007). Conversely, notable differences between seeds stored at 24, 4, and −20 °C (untreated) and those subjected to cooling methods and storage at −20 and −80 °C emphasised the impact of freezing methods on seed longevity and quality. Particularly, the significant difference in germination % observed in seeds rapidly frozen in LN slurry and stored at −80 °C (94 %) underscores the

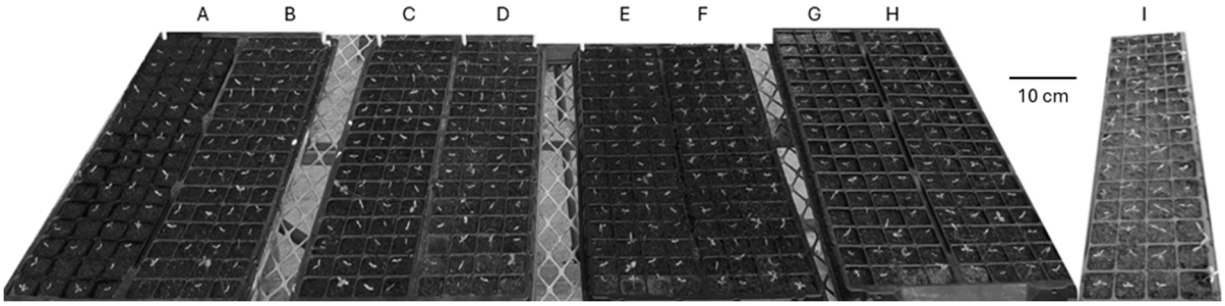

Fig. 2. Germination of *A. dubius* seeds in seedling trays 1-week post-sowing following multiple storage methods (A: 24 °C, B: 4 °C, C: −20 °C, D: −80 °C, E: Mr Frosty® cooling then −20 °C, F: LN slurry then −20 °C, G: Mr Frosty® cooling then −80 °C, H: LN slurry then −80 °C, I: unaged (control)).

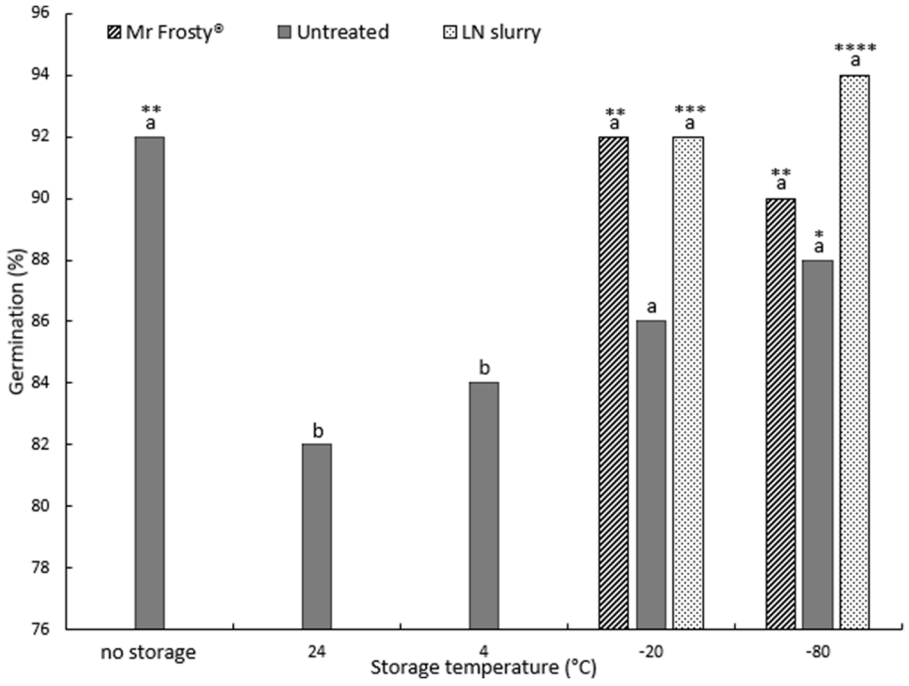

Fig. 3. Germination % of *A. dubius* seeds after 4 years of storage compared to the control (not stored). Different letters indicate statistically significant differences from the control and increasing * denote greater differences from the seed lot stored at 24 °C (Chi-square test; $p < 0.05$), $n = 64$.

potency of this technique in maintaining seed viability, surpassing traditional cold storage at 4 (84 %) and −20 °C (86 %). However, this method is more complex and requires more specialised equipment. The similarity in germination % between untreated seeds stored at −20 °C (86 %) and the control presents a compelling case for the efficacy of this storage method in preserving *A. dubius* seed viability because it is relatively more accessible and cost-effective than other, more sophisticated methods.

The rapid freezing process in LN has been shown to preserve the cellular structures and biochemical integrity of seeds by minimising ice crystal formation, preventing the physical disruption of cellular membranes and thereby maintaining the integrity of the seed tissue (Zeng et al. 2024). The observed success of rapid freezing can be attributed to the resilience of the meristematic tissues within seeds to extreme cold, which facilitated successful germination. This resilience is likely controlled by several factors, including cell membrane composition,

Table 1
Multiple growth characteristics of *A. dubius* seeds exposed to different cooling treatments and storage temperatures then germinated in seedling trays. Different letters indicate statistically significant differences among storage temperatures (lowercase letter) and cooling treatments (uppercase letters) (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 50$. FM = fresh mass.

Storage (°C)	Cooling treatment	Plant height (cm)	Number of leaves	Foliar FM (g)	Leaf area (cm^2)	Root length (cm)	Root FM (g)
Control	Control	$29.6^{aA} \pm 3.3$	$19.9^{aA} \pm 2.3$	$21.9^{aA} \pm 2.6$	$180.2^{aA} \pm 21.7$	$30.4^{aA} \pm 2.9$	$14.8^{aA} \pm 1.5$
24	None	$20.1^{cC} \pm 3.0$	$15.6^{cB} \pm 1.9$	$15.6^{dC} \pm 2.9$	$154.6^{bcB} \pm 28.5$	$19.7^{cC} \pm 2.8$	$10.3^{cB} \pm 2.3$
4	None	$21.2^{cC} \pm 2.7$	$18.1^{bB} \pm 2.2$	$18.6^{bC} \pm 2.5$	$156.3^{bcB} \pm 28.6$	$24.5^{bC} \pm 2.9$	$15.4^{aB} \pm 1.7$
−20	None	$23.1^{cC} \pm 2.8$	$17.3^{cB} \pm 1.7$	$18.8^{bcC} \pm 2.8$	$163.7^{bB} \pm 22.9$	$23.8^{bC} \pm 3.2$	$11.9^{bB} \pm 2.0$
−20	Mr Frosty®	$27.6^{bB} \pm 3.1$	$17.6^{cC} \pm 2.0$	$20.1^{bcB} \pm 2.7$	$184.6^{bA} \pm 26.6$	$27.4^{bB} \pm 2.7$	$15.2^{bA} \pm 1.6$
−20	LN slurry	$22.7^{bC} \pm 3.0$	$14.8^{cD} \pm 2.0$	$14.9^{bcD} \pm 2.1$	$140.4^{bC} \pm 21.7$	$21.9^{bB} \pm 3.1$	$10.0^{bC} \pm 2.0$
−80	None	$24.7^{bB} \pm 3.1$	$15.3^{dB} \pm 2.1$	$16.2^{cdC} \pm 1.9$	$158.6^{cB} \pm 24.9$	$24.5^{bC} \pm 2.8$	$12.0^{bB} \pm 1.7$
−80	Mr Frosty®	$27.4^{bB} \pm 2.6$	$12.9^{dC} \pm 2.2$	$19.8^{cdB} \pm 2.3$	$156.6^{cA} \pm 23.3$	$26.1^{bB} \pm 3.1$	$15.7^{bA} \pm 2.2$
−80	LN slurry	$23.4^{bC} \pm 2.9$	$12.1^{dD} \pm 2.2$	$14.7^{cdD} \pm 2.3$	$126.2^{cC} \pm 27.8$	$21.5^{bD} \pm 3.1$	$10.5^{bC} \pm 2.0$

antioxidant defence mechanisms, and gene expression. The long-term value of preserving seeds of ALVs, such as those of *A. dubius*, goes beyond the immediate considerations for maintaining seed viability.

Table 1 summarises the recorded growth characteristics of *A. dubius* seedlings subjected to various cooling and storage methods. The control group yielded the highest growth measurements. Mr Frosty® cooling significantly increased growth parameters in the treatment groups irrespective of storage temperature. This effect was particularly noticeable in the leaf area and root fresh mass (FM) measurements of plants grown from seeds stored at −20 and −80 °C. Seedlings from seeds stored at 4 °C had a root FM similar to the control and produced more leaves than seedlings grown from the other treated seeds. The LN slurry pre-storage treatment yielded seedlings with the lowest number of leaves, foliar FM, leaf area, root length, and root FM, suggesting that this specific cooling method had a detrimental effect on growth. Interestingly, there appeared to be a contrasting relationship between the observed high germination % and low growth characteristics exhibited by seeds cooled with the LN slurry. This pre-storage treatment possibly induced physiological stress on the seeds, negatively affecting growth parameters, but effectively maintained viability.

Some seeds can tolerate certain stress conditions during preservation without losing their ability to germinate, *i.e.* viability (Sahitya et al. 2018; Gama et al. 2021). Furthermore, germination is a fundamental process that, as shown by the majority of authors for a range of species, might be less sensitive to stress compared to subsequent growth (Galpaz and Reymond 2010; Patanè et al. 2012; Fu et al. 2019; Kong et al. 2020). Upon thawing, *A. dubius* seeds subjected to the LN slurry pre-storage treatment are likely to have activated adaptive mechanisms to cope with the stress induced by the treatment, including the activation of stress-responsive genes, leading to the synthesis of stress-related proteins that repair and protect cellular structures and maintain viability (Rajjou et al. 2008; Benítez-Rodríguez et al. 2013). Moreover, rapid cooling induces oxidative stress in cells due to the production of reactive oxygen species (ROS) (Coelho et al. 2018; Bailly 2019; Jurdak et al. 2020). The treated *A. dubius* seeds may have employed repair mechanisms to address damage incurred during the rapid cooling process and activated antioxidant defence mechanisms to neutralise ROS, minimising oxidative damage. These mechanisms could enhance germination potential while compromising other aspects of subsequent growth.

The activation of stress-responsive genes and synthesis of stress-related proteins require the allocation of energy and resources. When seeds invest resources in repairing cellular structures and combating oxidative stress, there is a trade-off with other essential processes, compromising the overall vigour of subsequent growth (Paul-Victor and Turnbull 2009; Notarnicola et al. 2022). The compromised allocation of resources and energy towards growth and development may lead to seedlings that are less robust, with slower growth rates and reduced capacity to compete for resources in the early stages of development. While necessary for protecting cellular structures, activating antioxidant defence mechanisms to neutralise ROS involves complex processes that compete with metabolic pathways essential for growth, compromising the efficiency of energy utilisation for growth-related processes (Akram et al. 2017). While stress adaptations enhance short-term survival, there might be implications for the long-term survival and reproductive success of the plant, influencing its ability to establish, compete, and reproduce. Seeds of *A. dubius* that underwent stress-induced adaptations during germination may become more sensitive when exposed to future environmental stresses, such as drought, salinity, competition, and nutrient deficit.

Preservation of whole seeds is preferred in cases where plant propagation and biodiversity conservation are prioritised, representing a fundamental component of plant genetic resources (Lynch et al. 2013; Moraes et al. 2019; Panis et al. 2020). Moreover, the benefits of seed storage, such as safety, minimal space requirements, and lower costs, further emphasise the significance of preserving seeds (Figueiredo et al. 2021). Conversely, preserving nodal explants and shoot tips is necessary for conserving genetic fidelity and facilitating vegetative propagation, including those species that are threatened or have limited seed availability (Engelmann 2011; Tavazza et al. 2013; Bradaï and Sánchez-Romero 2021; Popova et al. 2023).

3.2. Node cryopreservation

Representative specimens of micropropagated *A. dubius* nodal explants exposed to multiple cooling and storage protocols are shown in Fig. 4. The % of preserved *A. dubius* nodal explants developing at least 1 shoot (shoot development %) and the total number of new shoots produced per explant are shown in Fig. 5. Cooling explants with Mr Frosty®, followed by storage at −196 °C, was the only method that yielded a similar shoot development % to the control nodes, which were not cold stored. Furthermore, only direct LN immersion followed by −20 and −148 °C storage yielded significantly lower shoot numbers than the control. Interestingly, there appeared to be a correlation between shoot development % and the number of shoots in all treatments, except for the explants stored at −20 °C, which showed high deviation and low shoot development %. More new shoots often correlated with enhanced shoot development %, indicating a positive influence on shoot regeneration capacity. Untreated nodes were nonviable following storage at −148 and −196 °C.

Table 2 lists additional growth parameters of micropropagated nodal explants. As expected, the control, which was not stored and did not undergo any cooling treatments, yielded the highest growth parameters throughout micropropagation and subsequent acclimatisation. Mr Frosty® cooled explants yielded statistically similar shoot elongation rates to the control (2.4 ± 0.5 cm/week) when paired with storage at −20 (2.2 ± 0.6 cm/week), −80 (2.5 ± 0.4 cm/week), −148 (2.4 ± 0.5 cm/week), and −196 °C (2.4 ± 0.6 cm/week). Mr Frosty® cooled explants also achieved similar root elongation rates to the control (1.5 ± 0.6 cm/week) when stored at −148 (1.5 ± 0.4 cm/week) and −196 °C (1.5 ± 0.4 cm/week). None of the stored explants yielded similar foliar FM to the control (50.3 ± 5.1 g). Among the stored explants, those that produced the highest foliar FM were cooled by Mr Frosty® and LN immersion then stored at −20 (45.2 ± 5.1 g), −80 (45 ± 5.9 g), −148 (45.3 ± 4.1 g), and −196 °C (45.3 ± 5.8 g), and −20 (42.3 ± 4.4 g), −148 (45.0 ± 7.0 g), and −196 °C (44.4 ± 7.6 g), respectively.

Root elongation and foliar mass are widely recognised as integrative indicators of plantlet vigour following cryopreservation. In the presently tested *A. dubius* genotypes, preservation of root elongation rates equivalent to the control under Mr Frosty® cooling and subsequent storage at −148 and −196 °C, demonstrated that controlled-rate cooling can effectively protect the meristematic tissues responsible for below-ground development. By contrast, direct immersion in LN or LN slurry consistently impaired root elongation (<1.0 cm/week), suggesting that ultra-rapid cooling exposes basal tissues to mechanical injury from ice crystals and osmotic stress, thereby limiting root growth potential. This pattern is consistent with general observations across freezing stress literature, including another Amaranthaceae species, *Spinacia oleracea*, where successful post-cryopreservation recovery required protocols that balanced dehydration and cooling rates to avoid root stunting (Min et al. 2019; Ambroise et al. 2020).

Foliar FM offered a complementary measure of stress impact despite all stored explants exhibiting lower FM than the untreated control. This finding is representative of a recurring theme in leafy vegetables, where photosynthetically active tissues are highly sensitive to physical and oxidative damage, disrupting chloroplast integrity during cold storage (Liu et al. 2018; Ren et al. 2021). Such outcomes emphasise that while shoot regeneration and survival are immediate post-thaw indicators, biomass recovery is a longer-term and more stringent test of functional integrity. Thus, maintaining post-thaw vigour requires strategies that minimise both lethal and sub-lethal damage to photosynthetically and metabolically active tissues. Regardless, explants cooled with Mr Frosty® consistently approached the control values, demonstrating a

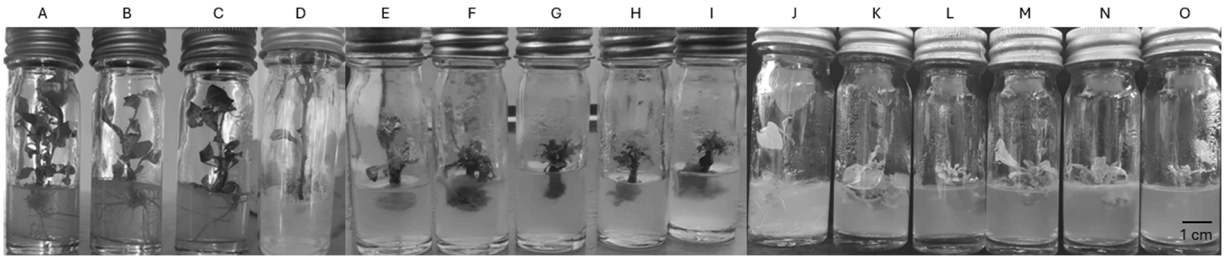

Fig. 4. Shoot development of *A. dubius* nodal explants exposed to various freezing and storage protocols (A: control, B: Mr Frosty® cooling then −196 °C, C: Mr Frosty® cooling then −148 °C, D: Mr Frosty® cooling then −80 °C, E: Mr Frosty® cooling then −20 °C, F: LN immersion then −196 °C, G: LN immersion then −148 °C, H: LN immersion then −80 °C, I: LN immersion then −20 °C, J: LN slurry then −196 °C, K: LN slurry then −148 °C, L: LN slurry then −80 °C, M: LN slurry then −20 °C, N: untreated then −80 °C, O: untreated then −20 °C).

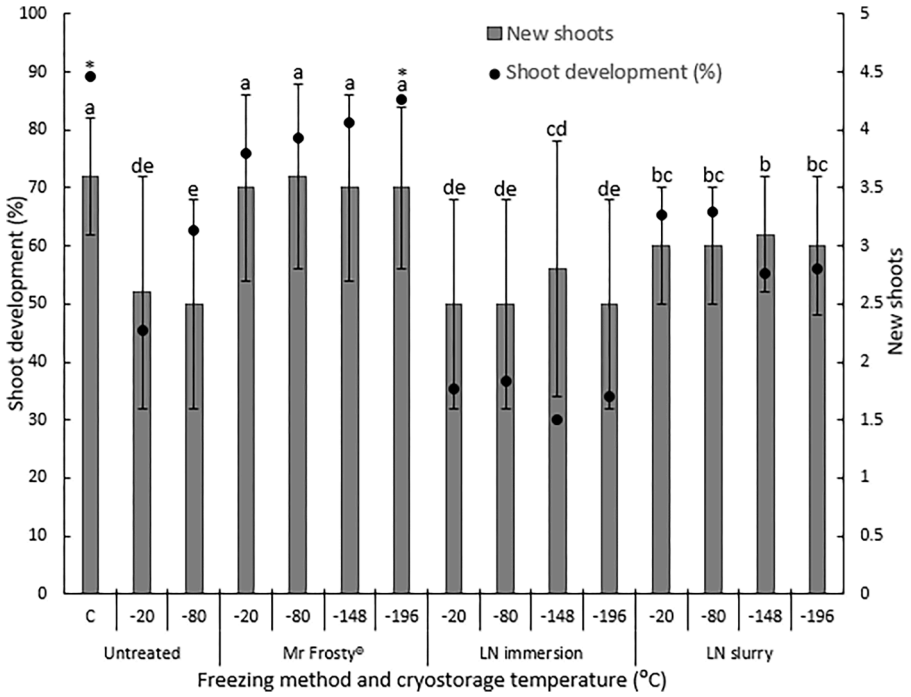

Fig. 5. The shoot development % and number of shoots forming from *A. dubius* nodal explants exposed to various freezing and storage protocols. Different letters indicate statistically significant differences in shoot numbers to the control (C) (Kruskal-Wallis test; $p < 0.05$), mean ± SD, $n = 135$. * Denotes similarity in shoot development % to the control sample (Chi-square test; $p < 0.05$), $n = 135$.

modest reduction in foliar FM. Controlled-rate cooling has been highlighted as one of the few consistently successful approaches across leafy vegetables, largely due to its role in reducing ice nucleation and allowing gradual water efflux. However, the ~10 % decline in FM relative to controls indicates residual stress costs that may compromise competitive ability or yield potential in field settings.

In the context of germplasm conservation, these results underscore that metrics such as root elongation and foliar mass should be prioritised alongside shoot regeneration when validating cryopreservation protocols. For crops like *A. dubius*, which are valued for both nutritional and agronomic resilience, protocols that preserve functional root and leaf development are essential to ensure that conserved accessions remain agronomically useful upon recovery.

The successful application of controlled-rate cooling, like Mr Frosty®, for the conservation of vegetative plant germplasm has also been documented in *Rubus* spp. (Uchendu et al. 2010), *Hypericum richeri* (Coelho et al. 2020), *Cannabis sativa* (Downey et al. 2021), and *S. lycopersicum* (Kulus 2019). The precise and gradual reduction in temperature afforded by Mr Frosty® offers several benefits, including consistency and reliability in the preservation of biological samples (Zhou et al. 2009), as well as the gradual removal of water from cells, which prevents the formation of deleterious ice crystals at the final storage temperature (Schulte and Reski 2004; Ishizaki et al. 2023). This method also facilitates the use of lower cryoprotectant concentrations to achieve intracellular freezing, thereby minimising toxicity and further enhancing viability (Jiang et al. 2004; Naaldijk et al. 2013).

Research into the molecular mechanisms governing shoot regeneration following cryopreservation in Amaranthaceae is sparse. However, in the model plant, *Arabidopsis thaliana*, this process involves a complex interplay of transcription factors, hormonal signalling, and gene expression regulation (Iwase et al. 2017; Zhang et al. 2017; Yang et al. 2022). In the context of the present study, the insights derived from studies in model plants like *A. thaliana* can offer parallels to reference and enhance the understanding of the molecular mechanisms involved in the shoot regeneration of *A. dubius*, presenting promising new avenues for future research.

3.3. Genetic fidelity of the salinity stress response post-cryopreservation

The relative expression (RE) of a previously documented NHX1-like gene was used to assess the effect of the tested cooling and storage protocols on the genetic fidelity of this salinity-responsive gene in a

Table 2
Growth parameters of micropropagated *A. dubius* nodal explants excised from seedlings grown from preserved seeds. Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 135$. FM = fresh mass.

Cooling method	Storage (°C)	Shoot elongation (cm/week)	Root elongation (cm/week)	Foliar FM (g)
Control	Control	$2.4^{ab} \pm 0.5$	$1.5^{a} \pm 0.6$	$50.3^{a} \pm 5.1$
None	−20	$1.3^{i} \pm 0.7$	$0.7^{efg} \pm 0.3$	$39.6^{d} \pm 6.4$
None	−80	$1.6^{gh} \pm 0.5$	$0.9^{cd} \pm 0.4$	$40.8^{d} \pm 7.2$
Mr Frosty®	−20	$2.2^{bcd} \pm 0.6$	$1.2^{b} \pm 0.6$	$45.2^{b} \pm 5.1$
Mr Frosty®	−80	$2.5^{a} \pm 0.4$	$1.1^{bc} \pm 0.5$	$45.0^{bc} \pm 5.9$
Mr Frosty®	−148	$2.4^{abc} \pm 0.5$	$1.5^{a} \pm 0.4$	$45.3^{b} \pm 4.1$
Mr Frosty®	−196	$2.4^{abc} \pm 0.6$	$1.5^{a} \pm 0.4$	$45.3^{b} \pm 5.8$
LN immersion	−20	$1.0^{i} \pm 0.5$	$0.9^{de} \pm 0.4$	$42.3^{bcd} \pm 4.4$
LN immersion	−80	$1.4^{hi} \pm 0.6$	$0.8^{def} \pm 0.3$	$42.1^{cd} \pm 6.0$
LN immersion	−148	$1.5^{hi} \pm 0.4$	$0.6^{g} \pm 0.3$	$45.0^{bc} \pm 7.0$
LN immersion	−196	$1.4^{hi} \pm 0.3$	$0.6^{fg} \pm 0.3$	$44.4^{bc} \pm 7.6$
LN slurry	−20	$1.9^{ef} \pm 0.5$	$0.9^{d} \pm 0.4$	$39.8^{d} \pm 8.1$
LN slurry	−80	$1.8^{fg} \pm 0.6$	$0.8^{de} \pm 0.4$	$40.0^{d} \pm 9.1$
LN slurry	−148	$2.2^{cde} \pm 0.7$	$0.9^{d} \pm 0.5$	$40.3^{d} \pm 8$
LN slurry	−196	$2^{def} \pm 0.6$	$0.8^{de} \pm 0.4$	$39.8^{d} \pm 7.5$

salinity-tolerant *A. dubius* genotype (S4). Fig. 6 shows the electrophoresis gel of total RNA extracted post-acclimatisation from salt-stressed root tissue samples of S4 explants subjected to the various storage methodologies, wherein 2 distinct bands, representing 28S and 18S ribosomal RNA were visualised, indicating pure and high-quality RNA samples with minimal degradation. Table 3 summarises the NHX1-like

Table 3
The NHX1-like gene relative expression (RE) and foliar fresh mass (FM) of *A. dubius* following acclimatisation in response to multiple cooling and storage methods. Different letters indicate statistically significant differences (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, $n = 9$.

Cooling method	Storage (°C)	NHX1 0 mM NaCl (RE)	NHX1 400 mM NaCl (RE)	Foliar FM (g)
Control	Control	$1.7^{bcd} \pm 0.4$	$9.7^{bcd} \pm 0.6$	$51.9^{a} \pm 6.6$
None	−20	$2.7^{a} \pm 0.5$	$10.4^{ab} \pm 0.6$	$45.3^{abcd} \pm 5.3$
None	−80	$2.1^{abc} \pm 0.5$	$8.4^{defg} \pm 0.7$	$46.4^{abcd} \pm 2.6$
Controlled-rate	−20	$0.7^{e} \pm 0.4$	$7.2^{gh} \pm 0.8$	$51.7^{ab} \pm 5.7$
Controlled-rate	−80	$1.0^{de} \pm 0.6$	$6.9^{h} \pm 0.5$	$50.4^{abcd} \pm 2.9$
Controlled-rate	−148	$1.7^{bcd} \pm 0.2$	$10.2^{abc} \pm 1.3$	$46.0^{abcd} \pm 3.4$
Controlled-rate	−196	$1.8^{abcd} \pm 0.5$	$8.9^{cdef} \pm 1.2$	$48.0^{abcd} \pm 4.3$
LN immersion	−20	$2.3^{ab} \pm 0.7$	$9.5^{bcde} \pm 0.7$	$43.1^{d} \pm 2.9$
LN immersion	−80	$2.7^{a} \pm 0.4$	$8.3^{efg} \pm 0.6$	$48.1^{abcd} \pm 4.8$
LN immersion	−148	$1.1^{de} \pm 0.6$	$11.4^{a} \pm 0.9$	$44.5^{bcd} \pm 4.9$
LN immersion	−196	$1.2^{cde} \pm 0.5$	$7.8^{fgh} \pm 0.8$	$50.2^{abcd} \pm 3.1$
LN slurry	−20	$1.1^{de} \pm 0.4$	$9.3^{bcde} \pm 1.0$	$46.8^{abcd} \pm 5.3$
LN slurry	−80	$2.4^{ab} \pm 0.9$	$9.1^{bcde} \pm 0.8$	$43.4^{d} \pm 4.7$
LN slurry	−148	$1.5^{bcde} \pm 0.6$	$9.1^{bcde} \pm 0.7$	$43.8^{cd} \pm 4.6$
LN slurry	−196	$2.4^{ab} \pm 0.7$	$8.3^{efg} \pm 0.7$	$51.1^{abc} \pm 3.8$

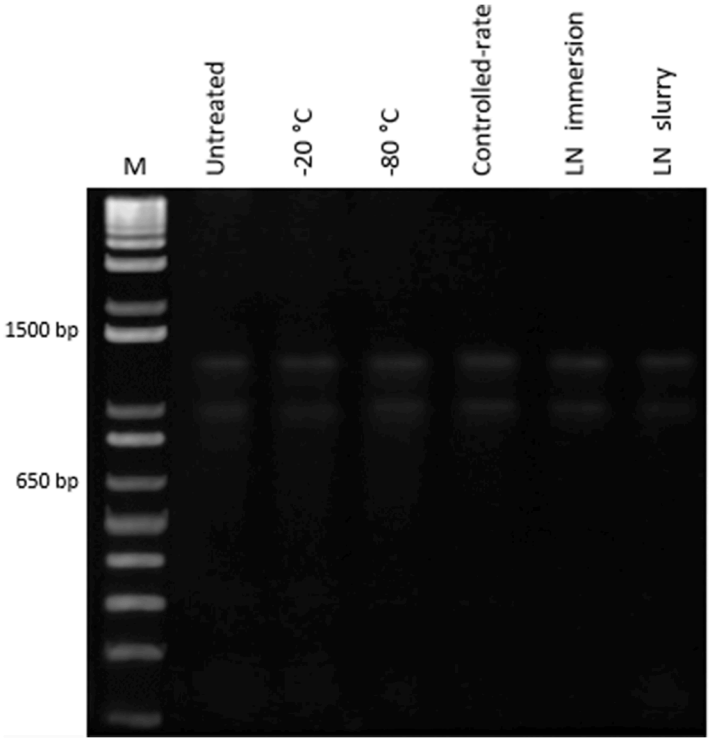

Fig. 6. Electrophoresis of 5 μg total RNA extracts from *A. dubius* roots exposed to salinity stress (400 mM NaCl) following multiple cryopreservation conditions. *M* = 1 Kbp marker (Invitrogen, USA).

RE normalised against MDH and foliar FM following acclimatisation of stored S4 nodal explants. Baseline RE of the NHX1-like gene was established by the non-stored samples and an untreated salinity stress group (0 mM NaCl) compared to the stored salinity-stressed samples exposed to 400 mM NaCl.

At 0 mM NaCl, the NHX1 RE was highest in untreated nodes stored at −20 and −80 °C, Mr Frosty® cooled nodes stored at −196 °C, LN immersed nodes stored at −20 and −80 °C, and LN slurry cooled nodes stored at −80 and −196 °C. These treatment groups resulted in upregulation of the NHX-like gene compared to the non-stored control group (1.7 ± 0.4) without exogenous salt application. When salt was added to the substrate (400 mM NaCl), untreated nodes stored at −20 °C, Mr Frosty® cooled nodes stored at −148 °C, and LN immersed nodes stored at −148 °C yielded specimens that exceeded the RE of the non-preserved control (9.7 ± 0.6). Overall, samples which resulted in diminished NHX1-like activity compared to the non-stored control were the untreated nodes stored at −20 and −80 °C, Mr Frosty® cooled nodes stored at −196 °C, LN immersed nodes stored at −20 and −80 °C, and LN slurry cooled nodes stored at −80 and −196 °C, indicating that these cooling and storage conditions reduced the genetic fidelity of the NHX1-like gene. Contrastingly, Mr Frosty® cooled nodes stored at −20, −80, and −148 °C, LN immersed nodes stored at −148 and −196 °C, and LN slurry-cooled nodes stored at −20 and −148 °C yielded plants that maintained or surpassed the RE of the non-preserved control, which suggest that these storage conditions upheld the genetic fidelity of the GOI in genotype S4.

These results are further supported by the foliar FM of salinity-stressed micropropagated nodes which were cooled by Mr Frosty® then stored at −20, −80, and −148 °C, immersed in LN then stored at-148 and −196 °C, and cooled in LN slurry then stored at −20 and −148 °C, yielding similar FM to the non-preserved control (51.9 ± 6.6 g). Interestingly, the following treatments also yielded similar foliar FM to the non-preserved control despite exhibiting decreased NHX1 activity: direct storage at −20 and −80 °C, Mr Frosty® cooling followed by storage at −196 °C, LN immersion followed by storage at −80 °C, and LN slurry cooling and storage at −196 °C. This result coincides with the elevated RE observed in samples not exposed to salt stress (0 mM NaCl), suggesting that, in addition to salinity tolerance, the putative NHX1 gene also influences *A. dubius* growth in response to storage-related stresses. The elevated NHX1 RE and consistency in foliar FM across diverse preservation treatments suggest that this gene mitigates storage-related stresses during plantlet regrowth – a result that has not been previously documented. Furthermore, the connection between elevated RE in samples which were not exposed to salt stress (0 mM NaCl) and the observed growth response further indicated that NHX1 may act as a modulator of growth in stressful environments. However, due to the underutilised and orphan crop status of the species of interest, there is not much literature to compare the present findings.

Broadly, The NHX1 gene, known for its role in ion transport and cellular homeostasis (Barragán et al. 2012; Chanroj et el. 2012), may influence plantlet vigour post-storage. However, this interaction has not been reported, presenting an avenue for future research. Ionic homeostasis is essential for osmotic regulation, ensuring that plant cells can control water uptake, thus maintaining turgor pressure and contributing to the overall cellular hydration and structural integrity of plantlets (Häussinger et al. 1996; Ritz et al. 2003). Additionally, cryopreservation procedures often subject plant tissues to various stresses, including dehydration and osmotic stress (Cantrel et al. 2011; Ren et al. 2021). NHX1, by facilitating ion transport and cellular water balance, may mitigate the impact of these stresses, contributing to the recovery of cell turgor and preventing cellular damage.

Furthermore, NHX1 activity is intertwined with hormonal signalling pathways, including those associated with abscisic acid (ABA) and auxins (Sun and Zhou 2017; Sardans and Peñuelas 2021). The influence of NHX1 on these pathways post-cryopreservation may contribute to optimal hormonal balance, promoting vigorous shoot and root development. Proper ion transport facilitated by NHX1 can enhance nutrient uptake by plant cells. Improved nutrient availability contributes to the vigour of plantlets by supporting metabolic processes, energy production, and overall growth. Understanding the intricate mechanisms by which NHX1 influences *A. dubius* plantlet vigour post-storage requires further investigation. Integrating more molecular studies, including whole-transcriptome expression profiling, hormone quantification, and cellular imaging, can provide deeper insights into the specific pathways and processes through which NHX1 contributes to the recovery and vigour of plantlets following cryopreservation.

The varied effects of cryopreservation treatments on gene expression are well documented and have been shown to affect gene expression patterns in *Solanum tuberosum* (Seo et al. 2018), *S. lycopersicum* (Kyu et al. 2019), *Hosta capitata* (Pe et al. 2021), *Olea europaea* (Bradaï and Sánchez-Romero 2021), and *A. thaliana* (Li et al. 2013; Gross et al. 2016). However, it is often ambiguous if those effects depend on other factors like regeneration conditions and specific genotypes. Moreover, other studies have documented genetic changes post-cryopreservation resulting from tissue culture conditions rather than cryopreservation (Castillo et al. 2010; Coste et al. 2015; Lee et al. 2021). Therefore, it cannot be conclusively said that the NHX1-like activity observed in the present study was solely due to preservation, micropropagation, or a combination thereof, necessitating further research.

4. Conclusion

The investigation into the long-term storage effects on *A. dubius* seeds yielded best practices for preserving seed viability over extended periods (4 years). Notably, no statistically significant differences were observed between the unaged control and seeds stored at −20 and −80 °C for 4 years, suggesting the effectiveness of these methods in arresting seed deterioration and maintaining viability similar to unaged seeds, thus presenting a compelling case for the efficacy of this storage method in maintaining *A. dubius* seed viability. The findings from the various cooling and storage methods presently tested on *A. dubius* nodal explants offer practical methods for preserving germplasm with enhanced shoot regeneration capacity. Among the tested methodologies, controlled-rate cooling followed by storage at −196 °C emerged as the sole method yielding shoot development percentages akin to the non-cryostored control nodes.

The findings reported herein establish a foundation for refining preservation protocols, guiding the selection of optimal storage conditions necessary for preserving *A. dubius* seeds and nodal explants. These findings provide knowledge for maintaining *A. dubius* seed viability, ensuring the preservation of genetic diversity and opportunities for enhancing agricultural productivity, and ensuring that unique, and often region-specific, genetic traits are conserved, such as potential resistance to pests, diseases, or environmental stresses (Linguya et al. 2015). Beyond agricultural applications, the ability to store *A. dubius* seeds has implications for ecological restoration efforts, as many ALVs have vital roles in local ecosystems, contributing to soil health and ecosystem stability (Maseko et al. 2017), thus enabling the conservation and potential reintroduction of these species in degraded or disturbed areas, supporting ecological restoration initiatives and promoting biodiversity conservation.

CRediT authorship contribution statement

Ashiq Haripershad: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Muhammad Nakhooda:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Shakira Shaik:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

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