Vitrification-cryopreservation procedure causes physiological and histology injuries to dormant buds of Codonopsis pilosula (Franch.) Nannf. while all maintain genetic integrity in cryo-derived plantlets

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Vitrification-cryopreservation procedure causes physiological and histology injuries to dormant buds of Codonopsis pilosula (Franch.) Nannf. while all maintain genetic integrity in cryo-derived plantlets

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Abstract

Vitrification-based cryopreservation exhibits considerable potential for the large-scale preservation of plant genetic resources. This study investigated the physiological and histological alterations in dormant buds of *Codonopsis pilosula* (Franch.) Nannf. during cryopreservation. At the loading stage, superoxide dismutase (SOD) activity and proline content were significantly elevated, whereas catalase (CAT) activity, peroxidase (POD) activity, ascorbate (ASA) content, and reduced glutathione (GSH) content were markedly reduced. Treatment with Plant Vitrification Solution 2 (PVS2) and freeze-thawing resulted in decreased SOD activity but exerted minimal effects on other aforementioned indicators. Plasmolysis was observed following the loading treatment and was further exacerbated after PVS2 exposure. Surviving cells were primarily localized in the apical meristem, leaf primordia, and upper region of the bud axis. Cooling in liquid nitrogen (LN) and subsequent freeze-thawing caused extensive damage to cells in the outer and lower regions of the bud axis.Cryopreserved shoot tips exhibited a high survival rate without detectable phenotypic or genetic variations. These findings enhance the understanding of the mechanisms underlying dormant bud cryopreservation and provide a basis for optimizing plant cryopreservation protocols.

Key message

This study investigated the physiological and histological alterations in dormant buds of *Codonopsis pilosula* (Franch.) Nannf. during cryopreservation, and identified the genetic stability of regenerated plants.

Keywords Antioxidants; Cryoinjury; Cryopreservation; Dormant bud; Genetic stability; Histology

1 Introduction

Cryopreservation refers to a technique for the long-term preservation of cells, tissues, and organs at ultra-low temperatures (-196°C). At this temperature, biochemical metabolic processes and cell division are completely arrested, enabling indefinite preservation of biological materials while maintaining high levels of plant genetic stability(Benelli et al. 2021; Matsumoto et al. 2014; Panis et al. 2020). Currently, efforts have been made to develop cryopreservation protocols for nearly all major agricultural and horticultural crops. A variety of plant tissues, including shoot tips, dormant buds, calli, embryonic axes, seeds, pollen, and root tips, have been successfully cryopreserved(Benelli 2021; Kaviani and Kulus 2022). Despite extensive research, the routine application of cryopreservation remains limited due to its dependence on species and explant type. This necessitates the optimization of protocols for almost every species, and even every genotype(Panis 2019). The development and optimization of protocols represent the most labor-intensive phase in cryopreservation research. Adapting cryopreservation protocols to diverse species thus poses a significant challenge.

Dormant buds possess inherent advantages, including natural cold tolerance and the ability to regenerate post-cryopreservation. Slow-freezing-based cryopreservation of dormant buds is one of the earliest methods employed for germplasm conservation . However, the slow-freezing technique requires expensive controlled-rate freezers, which limits its widespread application(Engelmann 2004; Kaczmarczyk et al. 2012; Mazur 1984). In recent years, vitrification has emerged as the dominant method for plant cryopreservation. This approach involves treating plant explants with cryoprotectant solutions to remove freezable water from cells(Benelli et al. 2021; El Merzougui et al. 2023). Vitrification-based cryopreservation is characterized by simplified operation, suitability for preserving larger organs with higher post-cryopreservation survival rates, and no requirement for expensive equipment(El Merzougui et al. 2023; Liu et al. 2022). Consequently, this method serves as a promising alternative to the slow-freezing technique for the long-term conservation of dormant bud germplasm(El Merzougui et al. 2023; Liu et al. 2022; Salgotra and Chauhan 2023).

Vitrification-based cryopre-servation of dormant buds has achieved high regeneration rates in various plant species, including blackcurrant cultivars(Rantala et al. 2021), *Salix(*Jenderek et al. 2020*)*, cherry(Kovalchuk et al. 2014), persimmon(Benelli et al. 2013; Matsumoto et al. 2014), *Betula pendula* Roth(Ryynänen and Aronen 2005), *Astragalus membranaceus* (Fisch) Bge. var. mongholicus (Bge) Hsiaoand(Dong et al. 2022) and *Gentiana straminea(*Ling et al. 2012*)*. Therefore, in-depth investigation into the mechanisms underlying dormant bud vitrification-cryopreservation is of great scientific significance. Conventionally, shoot tips isolated from dormant buds are used as cryopreservation materials, which requires a large quantity of shoot tips. In our previous study, intact dormant buds of *C. pilosula* achieved high recovery rates following vitrification-based cryopreservation. Regenerated plantlets were successfully obtained via in vitro induction of multiple shoots, providing a robust experimental system and sufficient samples for further research.

Cryoinjury is a critical factor affecting explant survival during cooling. Damage induced by physical injury during excision, osmotic stress, chemical toxicity of PVS2, and exposure to ultra-low temperatures can severely impair biological materials. These adverse effects manifest as various forms of damage, including disruption of cell membrane stability, alteration of cellular structure, modulation of antioxidant enzyme activities, and initiation of reactive oxygen species (ROS)-mediated injury(Burkhan et al. 2022; Wen et al. 2012). Elucidating the mechanisms by

which dormant buds tolerate such injuries is essential for the widespread application of vitrification-cryopreservation. However, research in this area remains limited.

Accumulating evidence indicates that the overproduction of ROS may impair antioxidant defense systems, making it one of the primary causes of cryopreservation-induced injury(Ren et al. 2021). A study using qRT-PCR to analyze antioxidant gene expression and physiological changes in Arabidopsis seedlings demonstrated that seedlings germinated for 48 hours exhibited enhanced antioxidant mechanisms in response to ROS(Chen et al. 2015). which represents a defensive response to oxidative stress reported increased levels of ROS and malondialdehyde (MDA) during the cryopreservation of embryogenic calli of Agapanthus praecox(Zhang et al. 2015). Additionally, previous studies have suggested that stress-related genes and proteins are activated during cryopreservation(Volk et al. 2011).

Microscopic observation has been widely used to investigate structural changes in explants during cryopreservation of shoot tips, embryos, cell cultures, and hairy root cultures(Cordeiro et al. 2020). In a previous study, our research team established a mature vitrification-based ultra-low temperature preservation protocol for dormant buds of C. pilosula, achieving a survival rate of up to 74%. On this basis, the present study was designed to investigate the response of dormant buds to vitrification-cryopreservation stress by analyzing dynamic changes in the antioxidant system, proline content, and histological injury. Furthermore, key antioxidant markers and histological changes in cryopreserved explants were identified, providing insights into critical steps of the protocol and facilitating future optimization for specific explants. Additionally, the genetic stability of regenerated plants was evaluated to validate the efficacy of this method for long-term conservation.

2 Materials and Methods

2.1 Plant Materials

Two-year-old roots of *Codonopsis pilosula* (Franch.) Nannf., were harvested from Longxi County, Gansu Province on November 15, 2022, The roots were covered with moist soil and stored at 4°C. Dormant buds were excised from the roots for vitrification-cryopreservation experiments.

2.2 Vitrification-Cryopreservation of Dormant Buds

2.2.1 Sample Preparation at Each Stage of Vitrification-Cryopreservation:

Loading (LS): Dormant buds were placed in 2 mL cryovials and treated with 1.0 mL of loading solution (MS liquid medium supplemented with 2.0 M glycerol and 0.4 M sucrose) at 20°C for 20 minutes；

PVS2 treatment: After the loading stage, the loading solution was replaced with PVS2 (MS liquid medium containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide, and 0.4 M sucrose). Dormant buds were treated with PVS2 at room temperature for 5 minutes (designated as P5) or 60 minutes (designated as P60).

Rapid cooling and thawing: Dormant buds were first treated with loading solution for 20 minutes, followed by PVS2 treatment at room temperature for 5 minutes or 60 minutes. The buds were then rapidly immersed in LN and held for 60 minutes, followed by thawing in a 38°C water bath for 2 minutes. These two treatments were designated as D5 (5 minutes of PVS2) and D60 (60 minutes of PVS2), respectively.

Recovery culture: Dormant buds were cultured on MS medium supplemented with 0.3 mg/L 6-benzyladenine (BA), 0.1 mg/L α-naphthalene acetic acid (NAA), 3% (w/v) sucrose, and 0.8% (w/v) agar (pH 5.8).

2.2.2 The following control groups were included in the experiment

Direct LN immersion (ZT): Dormant buds were transferred to 2 mL cryovials, directly immersed in LN for 60 minutes, and then thawed.

Sprouting dormant buds (MF): After 5 months of storage, dormant buds broke dormancy and began to sprout (bud length approximately 2 cm).

Untreated control (CK): Dormant buds without any treatment.

3 Experimental Methods

3.1 Determination of Oxidative and Osmotic Stress-Related Indicators

Dormant buds were rinsed three times with ultrapure water, blotted dry to remove surface moisture, wrapped in aluminum foil, rapidly immersed in LN for 30 minutes, and stored at -80°C until analysis. For each indicator, 0.2 g of sample was weighed (three biological replicates per treatment).

The activities of antioxidant enzymes (CAT, POD, SOD) and the contents of antioxidants (ASA, GSH) and proline in dormant buds subjected to different treatments were determined according to the manufacturer's instructions. A superoxide anion assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to measure superoxide anion inhibition rate and production.

3.2 Histological Observation of Dormant Buds

Shoot tips excised from dormant buds (as described in Section 2.2) were used for histological analysis. Shoot tips were first fixed in formalin-acetic-alcohol (FAA) fixative (ethanol:formalin:acetic acid = 18:1:1, v/v/v) for 24 hours. Subsequently, the samples were dehydrated through a graded ethanol series (70%, 85%, 90%, 95%, and 100% ethanol). After dehydration, the samples were embedded in paraffin and sectioned into 5-μm-thick slices using a microtome (Leica RM2125, Germany). The sections were stained with 0.01% toluidine blue (TB) according to the method described by Sakai (1973) and observed under a light microscope (Olympus BX 41, Japan). Freshly excised shoot tips, shoot tips immediately immersed in LN, and shoot tips cultured for 1 day after LN treatment were used as negative controls.

3.3 Evaluation of Genetic Stability of Cryo-Derived Plantlets

3.3.1 Morphological Analysis

After cryopreservation, dormant buds were inoculated on recovery medium to induce cluster buds and regenerate plantlets (designated as single-bud clones). A total of 40 plantlets derived from 10 single-bud clones were used for genetic stability assessment. The cryo-derived plantlets were transplanted into pots filled with vermiculite and irrigated with 1/10 strength MS solution. Morphological traits, including stem color, leaf color, leaf epidermal hairs, stem diameter, number of internodes, internode length, plant height, and growth vigor, were observed and recorded.

3.3.2 DNA Extraction

Genomic DNA was extracted from 20 mg of fresh leaf tissue using a Plant Genomic DNA Kit (Tiangen Biotech Co., Ltd., China) according to the manufacturer's protocol. The extracted DNA was purified and quantified using a UV spectrophotometer.

The DNA samples were diluted to a concentration of 50 ng/μL with deionized water and stored at -20°C for subsequent analysis.

3.3.3 ISSR Analysis

Polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 12.5 μL of 2× TaqMix (including 0.25 μL Taq DNA polymerase, 2×PCR buffer, 0.4 mM dNTPs, 3.2 mM $MgCl_2$, and 0.02% bromophenol blue), 10 pmol of ISSR primer, and 50 ng of genomic DNA template. The PCR amplification program was as follows: initial denaturation at 94°C for 4 minutes; 45 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 2 minutes; and a final extension at 72°C for 10 minutes. The PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0.1% ethidium bromide and visualized under ultraviolet light.

3.3.4 Statistical Analysis

In cryopreservation experiments, each treatment was performed with three biological replicates, with at least 10 shoot tips per replicate (a total of 30 explants per treatment). Data were analyzed using Duncan's multiple range test, and mean comparisons were conducted using the least significant difference (LSD) test at a significance level of $p < 0.05$. For genetic stability assessment, ISSR profiles were manually scored to determine the presence (1) or absence (0) of each band. Bands with low intensity that were difficult to distinguish were excluded from the scoring.

4 Results

4.1 Response of the Antioxidant System to Vitrification-Cryopreservation Stress

SOD, POD, and CAT are key antioxidant enzymes that work synergistically to scavenge excess O_2^- and H_2O_2 in cells, maintaining ROS at physiological levels, preserving cell membrane integrity, and protecting plants against vitrification-cryopreservation stress. The dynamic changes in the activities of these three enzymes during cryopreservation were analyzed.

When dormant buds were treated with the loading solution (containing high concentrations of sucrose and glycerol), hyperosmotic stress was induced, leading to the production of O_2^-. Consequently, SOD activity was significantly increased, indicating that dormant buds synthesize large amounts of SOD to counteract hyperosmotic stress during the loading stage. However, SOD activity was significantly reduced in both P60 and D60 treatments, suggesting that SOD was consumed to mitigate hyperosmotic stress induced by PVS2 and freezing stress caused by LN. In contrast, CAT and POD activities decreased during the loading stage but showed no significant changes in P60 and D60 treatments. This phenomenon may be attributed to the high inherent CAT and POD activities in dormant buds collected in mid-November, which are adapted to the cold and dry climate and sufficient to cope with hyperosmotic stress induced by the loading solution (Fig. 1-A1, B1, C1).

No significant differences in CAT, POD, or SOD activities were observed between P5 and P60, or between D5 and D60 (Fig. 1-A2, B2, C2). This suggests that different durations of PVS2 exposure and subsequent freeze-thawing treatments induce similar levels of oxidative stress in dormant buds, and prolonged PVS2 exposure does not further affect the activities of these three enzymes. CAT activity in the ZT group was significantly lower than that in the CK group, while POD and SOD activities were significantly higher than those in the CK group. These results indicate that direct immersion in LN without PVS2 protection causes severe damage to dormant buds, triggering the overproduction of ROS, and that antioxidant enzymes primarily respond to stress during the loading stage (Fig. 1-A3, B3, C3).

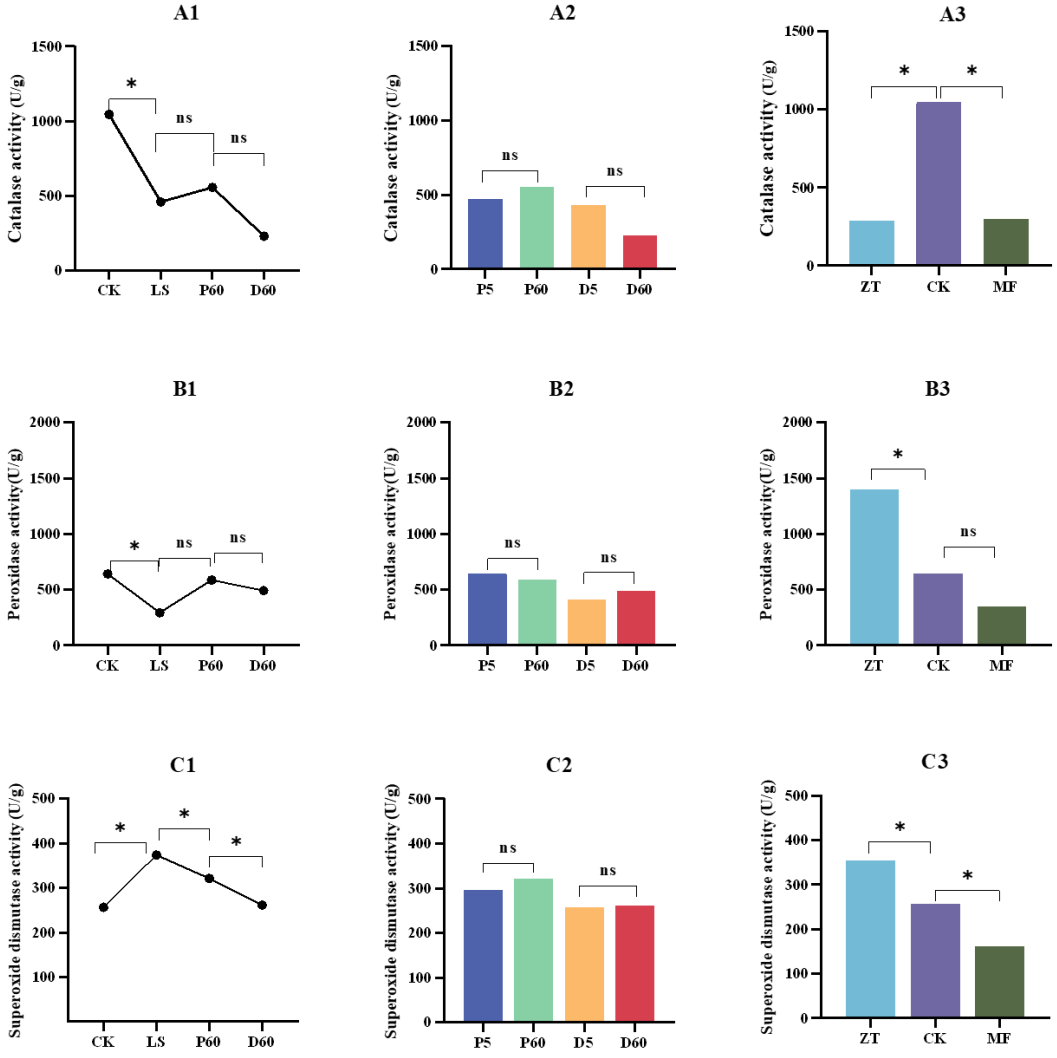


Fig. 1 Changes of CAT、POD and SOD activity during vitrification-cryopreservation processing. Capital letter: A CAT activity. B POD activity. C SOD activity. Numbers: 1 Various vitrification processing. 2 Various duration time of PVS2 treatment. 3 Untreated dormant buds, germinated dormant buds and dormant buds subjected to directly immersing into liquid nitrogen.

Note: * means significant at $P < 0.05$ level, ns means no significant difference, as indicated throughout the paper.

4.2 Changes in Antioxidant Content During Vitrification-Cryopreservation

Under oxidative stress, plants utilize not only antioxidant enzymes but also non-enzymatic antioxidants (such as ASA and GSH) to scavenge excess ROS. The dynamic changes in ASA and GSH contents during cryopreservation were consistent with the changes in CAT and POD activities: both ASA and GSH contents significantly decreased during the loading stage but showed no significant changes in P60 and D60 treatments (Fig. 2-A1, B1, C1).

No significant differences in ASA or GSH contents were observed between P5 and P60, or between D5 and D60. However, ASA and GSH contents were significantly reduced in the ZT group, which was consistent with the change in CAT activity (Fig.2-A2, B2, C2).The levels of ASA and GSH in LN decreased sharply after thawing compared to CK, which indicates that ZT treatment imposed great stress on dormant buds and induced excessive and lethal reactive oxygen species. The scavenging of these reactive oxygen species by ASA and GSH led to a considerable decrease in their levels. (Fig. 2-A3, B3, C3). Thus, increasing antioxidative activity of enzymes plays a significant role in the protection against cryogenic stress conditions.

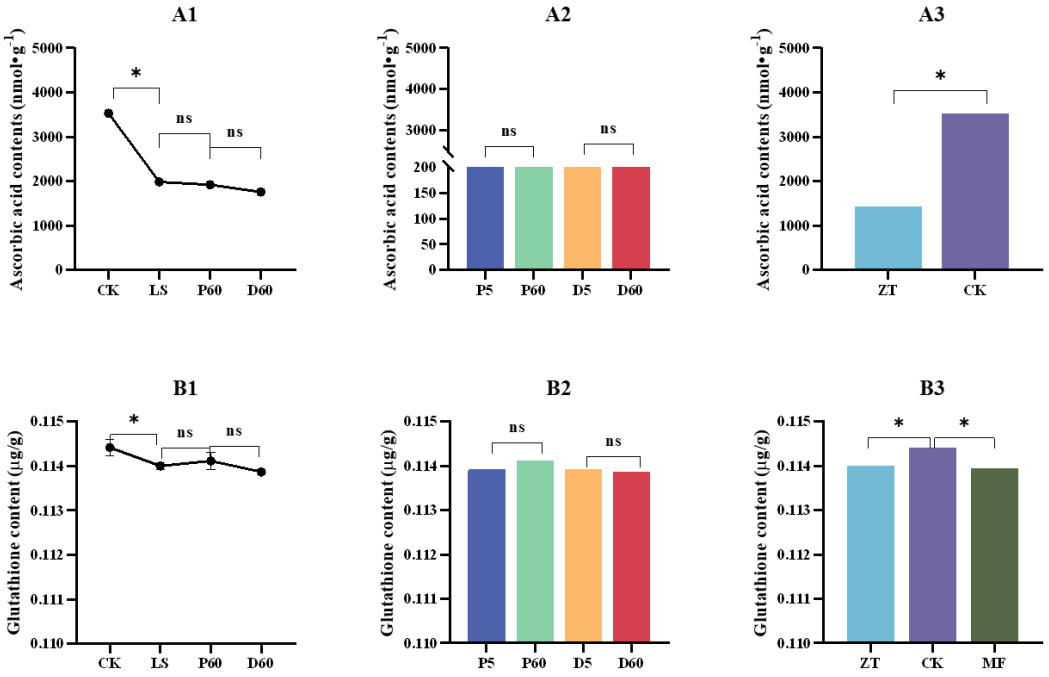


Fig. 2 Changes of ASA and GSH content during vitrification-cryopreservation processing. Capital letter: A ASA content. B GSH content. Numbers: 1 Various vitrification processing. 2 Various duration time of PVS2 treatment. 3 Untreated dormant buds, germinated dormant buds and dormant buds subjected to directly immersing into liquid nitrogen.

4.3 Changes of proline content during vitrification-cryopreservation

Proline could play a role in osmotic adjustment during drought stress, helping to alleviate and resist damage by increasing the concentration of the endocellular matrix. Plants accumulate various compatible solutes, such as proline, in response to cold and other osmotic stresses. The proline content in dormant buds increased significantly after LS treatment, indicating that proline responded to the hyperosmotic stress caused by LS. However, the difference was not significant after P60 and D60 treatment, which aligns with SOD levels (Fig.3-A1). The different PVS2 exposure times and their freezing-thawing treatments did not have a significant effect on proline content (Fig 3-A2 and Fig 3-A3).

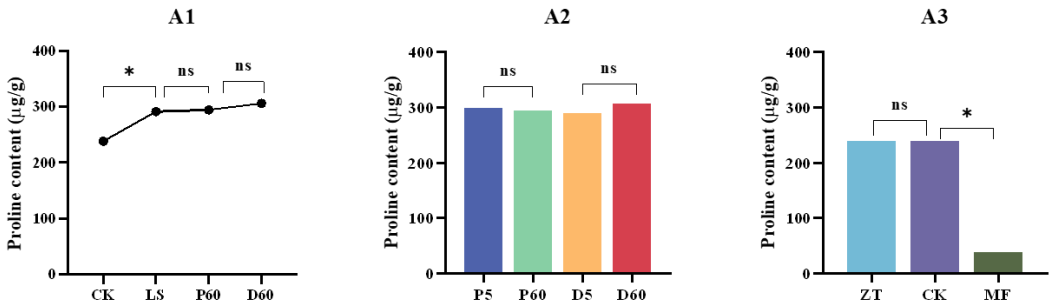


Fig. 3 Changes of proline content during vitrification-cryopreservation processing.
Capital letter: A proline content. Numbers: 1 Various vitrification processing. 2 Various duration time of PVS2 treatment. 3 Untreated dormant buds, germinated dormant buds and dormant buds subjected to directly immersing into liquid nitrogen.

4.4 Histological changes of dormant buds during vitrification-cryopreservation

The cells in the control group were tightly packed, with complete cellular structure, and clearly visible nucleus and nucleolus. The apical meristem cells and leaf primordium cells are tiny, virtually spherical with dense cytoplasm and a high nuclear-to-cytoplasm ratio (Fig. 4-B1). The approximately square cells in the upper part of the bud axis had a nucleus attached to the cell wall, symmetrically distributed with the nucleus of the neighboring cells (Fig. 4-B2). The cell volume of the lower part of the bud axis was greater than that of the upper part which nucleus was primarily dispersed in the center of the cell (Fig. 4-B3). Vascular tissue cells were long and thin, with a distinct nucleus and a high nuclear-cytoplasmic ratio.

Although the tissue and cell structure of dormant buds were intact following the LS treatment, slight plasmolysis was observed in cells of the lower part of the bud axis and vascular tissue (Fig. 4-C1, C2). Although the tissue cells still remained intact when dormant buds were subjected to P60 treatment, the plasmolysis was intensified.

The plasmolysis was evident in the apical meristem cells and leaf primordium cells. Serious damage was observed in dormant buds following treatment with D60. Cells located at the bud axis show uneven flocculus or translucency, indicating cell death (Fig. 4-D1, D2). Different from the cells in the lower part of the bud axist, the cells in the apical meristem and leaf primordium were still intact, with clear and round nuclei, and darker cytoplasm (Fig.4-E1, E2, E3). The above results illustrate that freezing in liquid nitrogen and thawing caused serious damage to the cells of the dormant bud axis, and the apical meristem and leaf primordium are the major locations of survival post-cryopreservation. When the dormant buds turned green in the recovery medium.

The cells had round nuclei and clear nucleolus. Slight plasmolysis was still observed (Fig. 4 F1). The degree of plasmolysis in the dormant bud cells after P5 treatment was milder than after P60 treatment, which indicated that with prolonged exposure to PVS2, the degree of cell dehydration increased (Fig. 5-A1, A2). Dormant bud cells treated with D5 and ZT were significantly damaged compared to those treated with D60. The cells in other regions were extensively injured, and the nucleus and cytoplasm appeared flocculated (Fig. 5-B1, B2, C1, C2). The apical meristem and leaf primordium cells of germinated buds were small and nearly round, with a large nucleoplasm ratio (Fig. 5-D1). The vascular tissue cells were elongated

strips with deeply stained cytoplasm (Fig. 5-D2). It demonstrates that the dormant buds were severely harmed after frozen in liquid nitrogen without adequate exposure to PVS2.

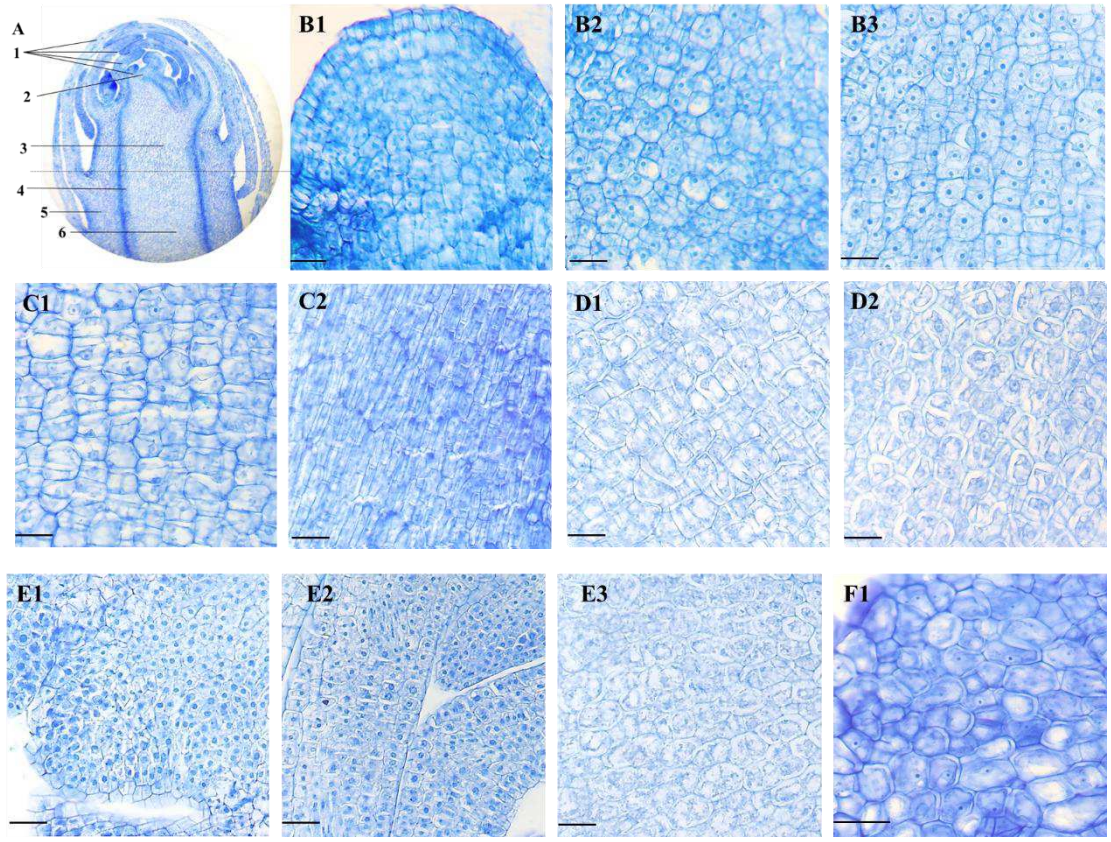


Fig. 4 Histological section of a C. pilosula untreated dormant buds(CK) and dormant buds subjected to CK, LS, P60, D60 treatment.

A Bulb morphology 1 Immature leaf; 2 Apical meristem; 3 Upper part of bud shaft; 4 Vascular tissues forming; 5 Outer layer of bud shaft; 6 Lower part of bid shaft. B CK B1: Apical meristem; B2 Upper part of bud axis; B3 Lower part of the bud axis. C Dormant buds subjected to LS treatment: C1 Upper part of bud axis; C2 Lower part of the bud axis. D: Dormant buds subjected to P60 treatment: D1 Upper part of bud axis; D2 Lower part of the bud axis. E Dormant buds subjected to D60 treatment: E1 Apical meristem; E2 Leaf primordia cell; E3 Lower part of the bud axis. F Restorative growth and turning green dormant buds. Bars=50μm.

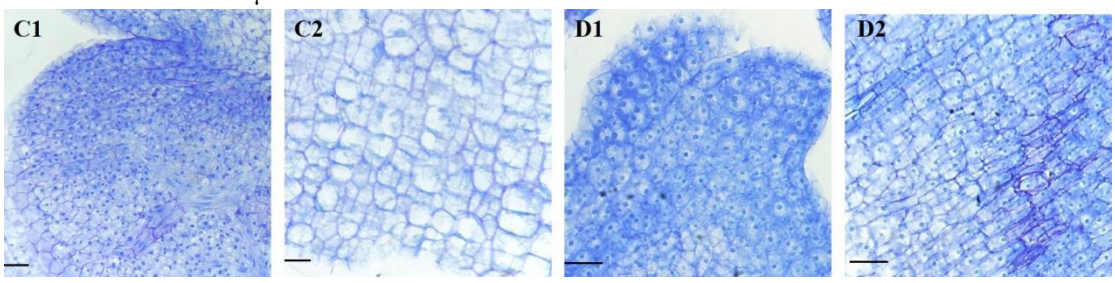


Fig. 5 Histological section of a C. pilosula germinated dormant buds (MF) and dormant buds subjected to ZT, P5, D5 treatment.

A1-A2 Dormant buds subjected to P5 treatment: A1Lower part of the bud axis； A2 Vascular tissue cell. B1-B2 Dormant buds subjected to D5 treatment: B1 Apical meristem; B2 Lower part of the bud axis. C1-C2 Dormant buds subjected to ZT treatment: C1 Apical meristem; C2 Lower part of the bud axis. D1-D2 MF treatment: D1 Apical meristem; D2 Vascular tissue cell. Bars=50μm.

5 The evaluation of genetic stability of cryo-derived plantlets

5.1 The morphology assessment

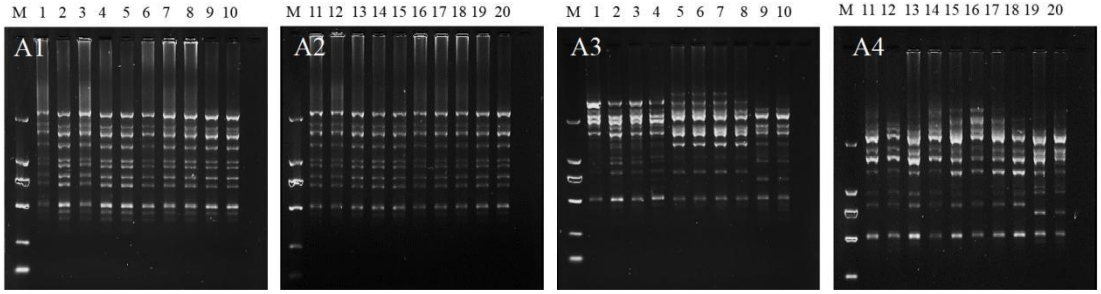
The morphology of 24 regenerated plants originating from 6 dormant buds after cryopreservation was examined. The results showed that the regenerants possessed green, heart-shaped leaves with serrated margins, green stems and white slender roots (Figs. 6 and Fig. 7), which were consistent in regenerated plants deriving from a single-bud clone (Fig. 8). However, the leaf epidermal hair in different clones exhibited diversity. Five clones all had short epidermal hair, except for one clone whose leaves were devoid of epidermal hairs.

5.2 ISSR-PCR molecular markers for evaluating genetic alterations

There was high consistency in the amplified band profiles among the plantlets within a single-bud clone (Fig. 8). A total of 1580 bands were amplified from 40 cryo- derived plants using these 5 primers. Results obtained in the present study indicate a low level of genetic variability within all analyzed samples.

Fig.6 Regenerated plants of C. pilosula dormant buds by vitrification-cryopreservation A.Regenerated plantlets B. Rooting plantlets

Fig.7 Transplanted plantlets via vitrification-cryopreservation of C. pilosula dormant buds



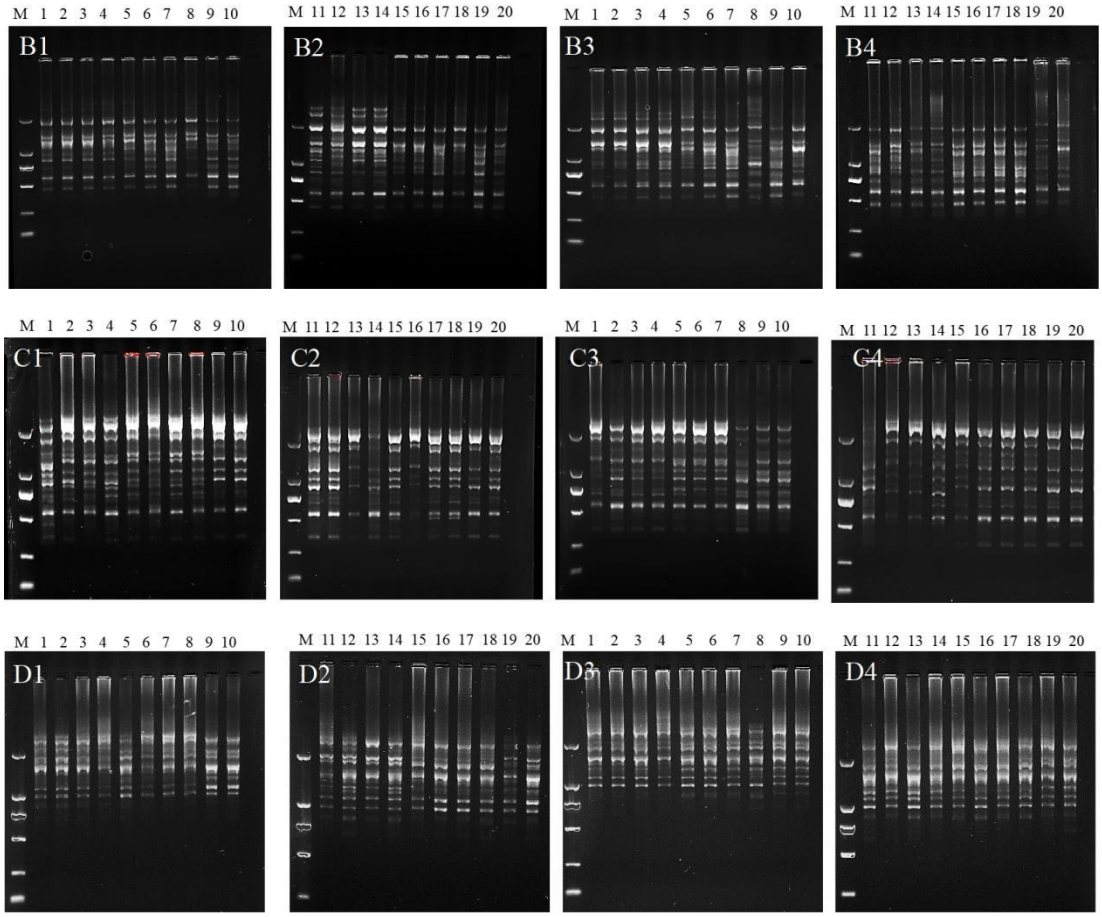


Fig. 8 Example ISSR band profiles of L. spectabilis 'Gold Heart' received as a result of electrophoresis of the DNA amplification products.

A1-A4: Primer 835 for PCR amplification of DNA from cryo-derived plants of C. pilosula. B1-B4 Primer ISSR-56 for PCR amplification of DNA from cryo-derived plants of C. pilosula. C1-C4 Primer 834 for PCR amplification of DNA from cryo-derived plants of C. pilosula. D1-D4 Primer 836 for PCR amplification of DNA from cryo-derived plants of C. pilosula. Outermost lanes (M) are DNA bp weight markers; 1, 2, 3, ... are the numbers of the plants: 1-4: Plantlets within a single-bud clone. 5-8: Plantlets from an individual bud clone. 9-10 and 19-20: Plantlets within another individual bud clone.

6 Discussion

6.1 Antioxidative systems respond positively to vitrification stress

Reactive oxygen species (ROS)-induced oxidative stress significantly hinders the progress of cryopreservation techniques for plant genetic resources(Funnekotter et al. 2017; Reed 2014; Ren et al. 2021). The vitrification-cryopreservation procedures significantly affected reactive oxygen species (ROS) levels, leading to the detection of H_2O_2, O^{2-}, and/or ·OH in *Arabidopsis* seedlings and embryogenic callus of *Agapanthus praecox* during loading and dehydration (Chen et al. 2015; Zhang et al. 2015) Antioxidant systems are essential for maintaining redox balance and defending against oxidative stress. In the current study, SOD activity and proline content increased significantly in loading treatment, whereas CAT and POD activity and also ASA and GSH content markedly decreased. PVS2 and freeze-thaw treatments markedly decreased SOD activity, with minimal effects on other indicators.

SOD is a vital antioxidant believed to decrease cellular oxidative stress, enhancing survival post-cryogenic exposure. Lynch et al. found that SOD activity in increase in pretreatment somatic embryos during olive tree cryopreservation(Lynch et al. 2011). Significantly higher SOD activity and ASA content were also detected in germinated rice embryos when treated with PVS2 solution in comparison with embryos treated only with the preculture and loading. In contrast, other studies have shown varying responses of SOD. For example, during the cryopreservation of zygotic embryos of *Amaryllis belladonna* L., increased superoxide production exacerbated cellular oxidative damage, leading to a significant reduction in embryo survival rates(Sershen et al. 2012). Additionally, in response to dehydration, SOD activity gradually decreased in the embryogenic callus of Agapanthus praecox, while CAT activity remained unchanged(Zhang et al. 2015).

Proline also plays a vital role in vitrification preservation. During the cryopreservation of *Dendrobium Sabin*, there was an initial increase in proline levels, followed by a subsequent decrease during the thawing process(James Antony et al. 2019). Likewise, there was a notable elevation in proline levels observed in correlation with extended preculture treatment periods during the cryopreservation process of gentian axillary buds(Suzuki et al. 2006). In our research, dormant buds were cryopreserved without prior preculture, resulting in a notable rise in proline content following the loading treatment, with no significant changes observed during the PVS2 treatment and thawing stages. In the cryopreservation process of *Dendrobium Sabin*, CAT activity showed a marked increase during the preculture phase, followed by a sharp decrease with no significant alterations in the subsequent stages(James Antony et al. 2019). However, in our study, CAT activity greatly decreased after loading treatment and with no obvious changes in PVS2 and thawing stages. Huang et al. observed that preculture led to a significant reduction in ASA content in 48-hour germinated rice embryos, whereas loading and PVS2 treatments substantially increased ASA levels(Huang et al. 2018). In the present study, ASA reduction was also detected at the first stage (loading) whereas no variation in PVS2 and thawing. Hence, the activity of antioxidant enzymes can be a crucial indicator for assessing cryopreservation tolerance, with responses varying based on the genotype.

6.2 Cell dehydration by LS and PVS2 to cope with cryopreservation stress

High concentration of sucrose and glycerinum in LS and PVS2 solutions results in hyperosmotic stress on cells, ultimately leading to cellular dehydration. Histological analysis in this study showed mild plasmolysis in dormant bud cells after LS treatment, intensifying with PVS2 treatment. Similar outcomes were noted during the cryopreservation process in shoot tips of peppermint(Volk and Caspersen 2007), Rubia akane(Salma et al. 2013), and in the roots of Cleomaceae(Salma et al. 2013). Furthermore, in the roots of *Rubia akane*, cells exhibiting meristematic traits, only underwent plasmolysis following exposure to the vitrification solution, similar with our research findings(Salma et al. 2013).

6.3 The main damage occurring in freeze-thaw steps

There is limited research on histological observation of cell survival after cryopreservation. In the study, loading did not visibly harm dormant buds compared to controls. However, PVS2 treatment severely dehydrated and deformed the protoplast of outer layer cells in the bud axis. Freezing and thawing are steps that cause major damage to bud axis cells, which lack a nucleus

or content, as seen in other plant species such as *Gentiana straminea* Maxim (Ling et al. 2012; Zhang et al. 2020) and citrus(Ding et al. 2008).

After the vitrification-cryopreservation procedure, the surviving cells were mainly found in the apical meristem, the youngest leaf primordia, and the upper part of the bud axis. Similar phenomena were also observed in the cryopreservation of apple(Feng et al. 2013), Rubus(Reed 1998), Dendrobium(Antony et al. 2014; Ching et al. 2012), Brassidium (Mubbarakh et al. 2014) and other plants. The reason lies in the small cells of the apical meristem, which possess a large nucleo-cytoplasmic ratio and dense cytoplasm without vacuole. These cells can achieve a higher survival rate after cryopreservation compared to mature cells with large vacuoles and a small nucleus(Volk and Caspersen 2007). Leaf primordia also exhibit similar characteristic, which ensure their survival after cryopreservation. The surviving tissues regrew into clusters of shoots, which are similar to peppermint shoot tips(Volk and Caspersen 2007).

6.4 Genetic stability of cryo-derived plants

Maintaining the genetic stability of the cryo-stored material is of utmost importance for plant germplasm conservation. Shoot tips are ideal candidates for the cryopreservation of most plants due to their unique advantages. They contain meristems that can germinate directly or produce multiple shoots, primarily regulated by hormone concentration. Progenies regenerated from shoot tips display genetic stability in comparison to the nonfrozen material in cryopreservation of *Dioscorea rotundata* Poir (Mandal et al. 2008)apple(Liu et al. 2008), sugarcane(Kaya and Souza 2017), *Dendranthema grandiflora* Tzvelev(Martín and González-Benito 2005), and grape(Mandal et al. 2008). Multiple shoot formation offers high proliferation efficiency and genetic stability, making it an attractive alternative for in vitro propagation(Zhang et al. 2020). Dormant buds have advantages because they contain shoot tips that can directly germinate or produce multiple shoots, mainly controlled by hormone levels. In the present study, no morphology and DNA changes were detected in the regenerants.

7 Conclusions

Vitrification-cryopreservation of dormant buds holds great promise for enhancing and expanding the use of cryogenic preservation. Results obtained here clearly demonstrate that loading and PVS2 cause progressive plasmolysis. This osmotic dehydration contributes to the vitrification process, ensuring that the samples are not seriously damaged after cryopreservation. Freezing and thawing are the main steps that cause major damage to the cells of the bud axis. No variations were observed in the genetic stability of cryo-derived plants, validating the use of this protocol as an efficient long-term conservation option for this species. The information on the antioxidant effects and histological changes in cryopreserved buds produced here offers insight into how tissues adapt to environment. This knowledge can help in developing simpler and standardized procedures that can be utilized by a broader range of institutions and laboratories, thereby enhancing preservation efforts.

8 Abbreviations

PVS (Plant Vitrification Solution); ROS (Reactive oxygen species); SOD (Superoxide dismutase); CAT (Catalase); ASA (Ascorbic acid); (GSH Glutathione)

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10 Data availability

The raw data supporting the conclusions of this article will be made available by the authors on request.

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12 Conflict of interest

We declare that this work was done by the authors mentioned in this article and that all responsibility for claims relating to the content of this article will be borne by the authors.

13 Author contributions

SLT conducted experiments, analyzed data, and wrote the manuscript. YHZ conceived and designed research and revised the manuscript. SFG CYH, and QYG conceived and designed research. JXW and SBS revised the manuscript. All authors have read and agreed to the published version of the manuscript.

14 Competing interests

The authors have no relevant financial or nonfinancial interests to disclose.

15 Ethics Declaration

This manuscript is not concurrently submitted to other journals and will not be during review; it is original, unpublished in any form or language, with prior research material reuse clearly disclosed to avoid text recycling, and free from "salami publishing" or secondary publication. All results are honest and accurate (no data fabrication/falsification/manipulation), follows discipline-specific data standards, with raw data stored for verification. We confirm no plagiarism—summarized content is properly cited, and a pre-submission check confirmed no issues. Citations are relevant, without excessive/improper self-citations or coordinated co-author self-citations. Author details and order are final, ICMJE-compliant, and we will promptly provide raw data etc for verification upon request.

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