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Vitrification-cryopreservation of dormant buds of Fritillaria przewalskii Maxim. and evaluation of its histology injuries and genetic fidelity of cryo-derived plants

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Abstract

Germplasm protection of an endangered plant *Fritillaria przewalskii* Maxim. is important to preserve genetic diversity, to store material for breeding. In the current study, A vitrifcation method was developed for cryopreserving dormant buds of *F. przewalskii*. Bulblets collected at July were stored at 4℃ for 3-4 months cold acclimation, 2-5 mm dormant buds dissected from the bulblets were pre-cultured on 0.5M sucrose 1/2 MS medium for 3 days. Then the dormant buds were treated with loading solution for 20 min at room temperature, dehydrated with vitrification solution 2 (PVS2) for 60 min, and finally directly plunged into liquid nitrogen. After rapid warming in water at 38°C, the dormant buds were directly plated on recovery medium without unloading. The recovery rate reached up to 93%. Successfully vitrified dormant buds developed bulblets within 5 weeks without intermediary callus formation. Freezing-thawing steps caused severe damage to the buds axis whereas cells in shoot apical meristem and leaf primordium were still intact and normal. Loading and appropriate PVS2 treatment resulted in hyperosmotic pressure leading to progressively cell plasmolysis which is beneficial to cell alive suffered ultra-low temperature. According to the morphology and the RAPD profiles of regenerated plants, no variation was found. As an superior cryopreserved material, dormant buds can assist in a faster and efficient development of new protocols or even in the creation of easy-to-use procedures.

1. Introduction

Fritillaria przewalskii Maxim. is a perennial herbal species in family Liliaceae, and it is one of the essential source of the traditional Chinese medicine "Chuanbei". Its dried underground bulbs has medical functions for clearing heat and moistening lung, relieving phlegm, and eliminating carbuncle (Chinese Pharmacopoeia Commission 2020). *F. przewalskii* is distributed to the western provinces of China, including southern Gansu, western Sichuan, eastern and southern Qinghai. It inhabits in alpine meadows or grasslands with elevations ranging from 2800 to 4400 meters (Chang et al. 2010). *F. przewalskii* prefers cool, shade and moisture environment, which limited the species distribution (Chen et al. 2020). The market demand is great due to its effectively therapeutic properties. However, the supply of *F. przewalskii* can’t catch up the market requirements because the species has the characteristic of slow-growing, long life cycle, and low yield under the natural condition (Carasso et al. 2011). At present, *F. przewalskii* in the medicinal market are mainly from wild plants, and the over-harvesting has led to a sharp decline of its resourses, which caused *F. przewalskii* listed as an endangered plant (Zhang et al. 2010). Therefore, artificial preservation and cultivation are imperative for the sustainable utilization of this medical herb species.

Cryopreservation, which cooling cells or whole tissues to ultralow temperatures often using liquid nitrogen (-196°C), halts all cellular divisions and metabolic processes, allowing theoretically unlimited storage under these conditions. (Benelli et al. 2009; Matsumoto et al. 2001). Vitrification-cryopreservation has become the predominant approach for long-term germplasm storage which include dehydrating the plant cells in high-concentration vitrification protective solution, followed by quickly solidifying cells in the liquid nitrogen, which make cells enter the vitrification state without time to form ice crystals (Chen et al.

2020). Therefore, vitrification-cryopreservation is considered as a simple, low cost, harmless method for the long-term conservation of plant germplasm resources (Choudhary et al. 2014; Gupta and Mir 2019).

Plant materials cryopreserved included pollen, seed, shoot tip, callus, suspension cells. Many of these plant materials can be difficult to cryopreserve due to their high water content, high levels of metabolism, and sensitivity to stress (Ren et al. 2013). Dormant buds undergo protective dehydration after cold-acclimation in winter, characteristic of low water content, low levels of metabolism and the strong frost resistance. Thus, the serious damage of dehydration and freezing can be avoided during vitrification-cryopreservation which can ensure high survival rate after freezing (Benelli et al. 2009; Uchendu et al. 2013; Matsumoto et al. 2015). It has established a large number of cryopreservation collections using dormant buds in the National Center for Conservation of Genetic Resources (NCGRP), the Institute of Plant Genetic Resources of Cornell University. However, all the practice used slow-freezing technique which relies on freeze-induced dehydration and primarily uses for cryopreservation of undifferentiated cultures and apices of cold-tolerant species, and requires the use of expensive controlled freezers, which limit its extensive utilization (Engelmann 2004). In this paper, we presented for the first time a protocol for vitrification-cryopreservation of *F. przewalskii* dormant bud and assess the histological changes in the process of vitrification-cryopreservation and the genetic fidelity of the cryo-derived plants.

2. Material and Methods

2.1 Explants source

Fritillaria przewalskii Maxim. bulbs were collected from Minxian County, Gansu province in July 2022, covered with moist soil and stored in a 4℃ germplasm cabinet.

2.2 Explants disinfection

F. przewalskii bulbs were gently cleaned with tap water using a soft brush, peeled off the outer scales, and dormant buds were disinfected with 75% ethanol for 30 second and 0.1% mercury chloride for 1 minute, finally washed with sterile water for 3 times and blotted on sterile filter paper for use.

2.3 Vitrification-cryopreservation

The dormant buds were placed in sterile cryovial, treated with loading solution (2 M glycerin, 0.4 M sucrose in MS liquid medium) (Sakai et al. 1990) at room temperature for 20 minutes, then dehydrated with PVS2 (30% w/v glycerol, 15% w/v ethylene glycol, and 15% w/v dimethyl sulfoxide in MS liquid medium with 0.4 M sucrose) (Sakai et al. 1990) or PVS3 (50% w/v glycerol, 50% w/v sucrose in MS liquid medium) (Nishizawa et al. 1993), and immediately submerged into liquid nitrogen. To evaluate the survival rate after cryopreservation, the cryovial were taken out from liquid nitrogen, and thawed in water bath at 38℃ for 2 minutes. Regeneration after post-cryopreservation was performed by in vitro culture on 1/2 MS media with 0.5 mg/L 6-BA, 0.1 mg/L NAA, 3% sucrose and 0.7% agar. During the first week the explants were maintained in the dark and then transfered to 12 hours photoperiod a day with 1500–2000 lx light intensity.

Effects of five factors of the vitrification-cryopreservation process on survival rate and regeneration rate were studied, i.e. the size of dormant buds [large (4–5 mm), medium(2–4 mm), small (1–2 mm)], low temperature treatment time before cryopreservation (2 months, 3 months, 4 months), PVS2 treatment time (30 minutes, 60 minutes, 90 minutes, 120 minutes), PVS3 treatment time (60 minutes, 90 minutes, 120 minutes, 150 minutes), loading and unloading (Have loading solution treatment is recorded as ' +ld ', Non-loading is ' -ld ', and have unloading solution is ' +ud '). Survival rate was recorded 7 days after post-culture and expressed as percentage of the total number of dormant buds turning green, while regeneration rate was defined as percentage of the total number of dormant buds regenerating into bulblets 5 weeks after post-culture.

2.4 Histological changes of dormant buds during cryopreservation

Based on the above optimized procedure, the dormant buds were collected at the following steps:

1. Control (CK+): Untreated dormant buds.
2. Negative control (CK-): Dormant buds were transferred to 2 mL cryovials and directly plunged into LN held more than 60 minutes followed by thawing.
3. Loading (LS): The dormant buds were immersed in 2 mL cryovials at room temperature for 20 minutes with a loading solution.
4. PVS2 (P_{60}): After loading dormant buds were treated with PVS2 for 60 minutes at room temperature.
5. Put into LN (LN_{60}): Dormant buds after dehydration in cryovials were rapidly plunged into liquid nitrogen (LN) and held for 60 minutes.
6. Thawing: Dormant buds after LN freezing rewarmed in 38℃ water bath for 2 minutes.
7. Recovery (R_{60}): The dormant buds were cultured on 1/2MS medium supplemented with 6-BA 0.5 mg/L, NAA 0.1 mg/L, 3% (w/v) sucrose, 0.7% (w/v) agar, and pH 5.8–6.0.

Dormant buds were fixed in FAA (ethanol:formalin:acetic acid = 18:1:1) solution for 24 hours, dehydrated through an incremental ethanol series (70%, 85%, 95%, and 100% ethanol) and finally embedded in paraffin. For light microscopy studies, 5 μm thick sections were cut using a Leica RM2125 microtome and stained with 0.01% toluidine blue (TB) (Li 2009). Untreated dormant buds served as a positive control, while those that were freshly excised, directly immersed in LN served as a negative control.

2.5 Evaluation of genetic stability in regenerated plants

2.5.1 Morphological markers

The morphological index of the regenerated bulblets and the original bulblets were observed, including color and shape of the bulblets and leaf shape and color of plantlets.

2.5.2 Genomic DNA isolation and RAPD markers

Genotype of the regenerated *F. przewalskii* was evaluated by random amplified polymorphic DNA (RAPD). DNA was extracted from 20 cryo-derived bulblets, which was derived from 5 dormant buds, using modified CTAB method (Coelho et al. 2014). DNA was purified and quantified using ultraviolet spectrophotometry. Each sample was diluted to 50 ng/μL in deionized water and stored at -20℃ until use. 20 primers were screened to select suitable primers for assessment of genetic stability of the regenerated bulblets. PCR was conducted in a reaction mixture containing 12.5 μL 2×Taq PCR Master Mix (with Dye), 1 μL of 10 μmol/L primer and 2 μL of 50 ng/μL DNA template in a final volume of 25 μL. PCR amplification was performed in a thermal cycler under the following reaction conditions: initial denaturation step for 4 minutes at 94℃, 38 cycles at 94℃ for 30 seconds, annealing at 40℃ for 60 seconds, and extension at 72℃ for 2 minutes followed by the final extension step at 72℃ for 8 minutes (Jiang et al. 2012). The PCR products were separated by electrophoresis in 1.5% agarose gel containing GoldView and the image was photographed under a gel image system. The molecular mass ladder DNA Marker DL2000 was used for estimating the size of the amplified products.

2.6 Statistical Analysis

In the cryopreservation experiments, at least 10 dormant buds were used in each of three replicates and all experiments in a total of 30 explants per treatment. The data were compared by Duncan multiple-range test, and comparisons between the mean values ere evaluated by the least significant different test at $p < 0.05$. In assessment of genetic stability, ISSR and profiles were manually scored for the presence and the absence of each band. Bands with low visual intensity that could not be readily distinguished were not scored.

3. Results

3.1 Factors affecting vitrification-cryopreservation of dormant buds

3.2.1 The treatment of PVS2 dehydration

No significant difference was found in survival rate of dormant buds post-cryopreservation by different PVS2 dehydration time, whereas regeneration rates exhibited difference (Fig. 2). The survival rate of dormant buds was not significantly varied among PVS2 duration from 30–120 minutes, but the regeneration rate was significantly decreased when the dehydration time is longer or shorter than 60 minutes. The best results were obtained with 60 minutes, which survival and regeneration rate is 47% and 43% respectively. It indicated dehydration duration is the key factor for dormant buds regeneration after cryopreservation.

3.2.2 Dormant buds size

The sizes of dormant buds (referring to length) significantly affected the survival and regeneration rates of post-cryopreservation. Survival and regeneration were both enhanced with increasing length of

dormant buds (Fig. 3). When the dormant buds were shorter than 2 mm, the survival and regeneration rates were the lowest. The highest survival and regeneration levels were 77% and 70% respectively when the length of dormant buds was 2–4 mm. However, when the length reached 4–5 mm, the survival and regeneration rate showed no difference from 2–4 mm. Thus, 2–5 mm long dormant bud is recommended for high regeneration of cryopreserved propagule.

3.2.3 Sucrose preculture

The effects of different sucrose concentrations and preculture time on the survival and regeneration rates of dormant buds after cryopreservation were compared with non-precultured control. Both survival and regeneration rates of dormant buds steadily and slightly reduced with the increase of sucrose concentration for 1 day preculture duration, while both rates increased with 0.3 M and 0.5 M sucrose and slightly decreased at 0.7 M sucrose for 3 days preculture (Fig. 4). The survival rates among treatments were not significantly different, but the regeneration rate in 0.7 M sucrose for 1 day were significantly lower than other treatments. In the control group, dormant buds had 77% survival rate and 67% regeneration rate, respectively, which were lower than other treatments except 0.7 M sucrose for 1 day. Dormant buds had the highest survival and regeneration rates, reaching to 93% at 0.5 M sucrose for 3 days, and improved the survival rate by 16% and the regeneration rate by 26%. Therefore, the optimal preculture conditions for dormant buds were 0.5 M sucrose for 3 days.

3.2.4 Loading and unloading

Non-loading had no obvious influence on the survival rate of dormant buds after cryopreservation compared with loading, whereas loading significantly improved the regeneration rate, which is higher than non-loading by 37%. (Fig. 5). The result suggested that dormant dehydrated by loading is crucial for avoiding severe damage to dormant bud and maintaining its viability in cryopreservation. Unloading is the routine step of vitrification-cryopreservation dormant buds with unloading resulted the higher survival and regeneration rates, which is 83% and 77%, respectively, but the difference was not significant compared with non-unloading. Thus, loading is an essential step of cryopreservation of dormant buds of *F. przewalskii*, while unloading is not necessary for cryopreservation. Removing the unloading step can simplify cryopreservation procedures and reduce the cost.

3.2.5 The treatment of PVS3 dehydration

Dehydration time to PVS3 had a significant effect on cryopreservation of dormant buds (Fig. 6). The survival and regeneration rates of dormant buds were enhanced with the increasing of the PVS3 exposure time from 60–120 minutes, and then significantly decreased for 150 minutes PVS3 treatment, with the lowest survival rate and regeneration rate (43% and 30%), but the highest survival and regeneration rate (83% and 63%) were achieved in 120 minutes. It suggested that too long exposure time to PVS3 would cause excessive dehydration of dormant buds which can damage its vitality seriously and result the death of dormant buds post-cryopreservation.

3.2.6 Cold acclimation time

Cold acclimation is an extremely important factor influencing cryopreservation of dormant buds. With the increasing of cold acclimation time, the survival and regeneration rates of dormant buds enhanced remarkably (Fig. 7). The survival rate and regeneration rate of dormant buds were only 47% and 37% respectively, when the bulblets were processed at 4℃ for 2 months. However, when the enduration time added up to 3 months, the two indexes significantly increased to 30%. With the time prolonged to 4 months, there was no difference in the two indexes compared to 3 months. Therefore, keeping the bulbs of *F. przewalskii* at 4°C for 3–4 months before using them for cryopreservation will improve their recovery.

3.2 Recovery culture

The dormant buds as a slender conical shape was pale yellow before cryopreservation (Fig. 1a), while it became white post-cryopreservation (Fig. 1b). Within 7 days of cultivation, some buds gradually turned green (Fig. 1c, as indicated by white arrow) which were considered as viable, and others not turned green or brown were considered as dead (Fig. 1c, as indicated by black arrow). After 5 weeks of cultivation, the viable dormant buds produced 4–5 small, white, and spherical bulblets on the surface (Fig. 1d). Regenerated bulblets could propagate continuously by sublture (Fig. 1e) and when trasfered to rooting medium the bulblets could geminate and grow into plantlets (Fig. 1f).

3.3 Histological changes of the dormant shoots in the process of cryopreservation

Cells of the positive control dormant bud were characteristic of dense TB-stained, well-preserved cytoplasms and nucleoli (Fig. 8b1, b2) enclosed in the nucleus. The apical meristem cells and leaf primordium cells are tiny, virtually spherical with dense cytoplasm and a high nuclear-to-cytoplasm ratio. Nucleus and nucleolus were clearly visible (Fig. 8b1). The bud axis cells are approximately rectangular, the cells are large, the nucleus is located in the center of the cell, and there are one or more nucleoli in the nucleus (Fig. 8b2). In the dormant bud of negative control, the overall staining of cells was relatively light. A typical sign of ruptured plasma membranes was observed, the nucleus dissolves and the nucleolus was not visible (Fig. 8c1, c2). Although LS did not cause marked cell damage to shoot tips, slight plasmolysis was observed (Fig. 8d1, d2). When the buds were further treated with PVS 60 minutes the tissue and cell structure still remained intact while the plasmolysis intensified. The plasmolysis was greatest at the cells of the lowest half of the bud axis (Fig. 8e1, e2). Serious damage was caused by cooling in LN and thawing. In the cryopreserved cells located at the basal parts of the dormant bud (Fig. 8f1, f2) were found already dead, while cells located in the apical domes and in the leaf primordia were alive. When the dormant buds turned green in recovery medium, the complete cell structure with clear and visible nuclei in circular shape in the center of the cells, with nucleoli visible in a few cells (Fig. 8g1, g2).

3.4 Evaluation of genetic stability in regenerated plants

3.4.1 Morphological markers

The regenerated bulblets were not different from the original materials. Regenerated bulblets were composed of two scales, one large and the other small, and the small one was embraced by the large one, and there are dormant bud inside. Both scales were white in color and dense in texture. The leaf of regenerated plant was strip or strip lanceolate, green in color.

3.4.2 RAPD markers

Three RAPD primers were selected to genotype 20 random samples of the regenerated bulblets for detecting the genetic stability after cryopreservation. The results showed that three primers generated a total of 400 polymorphic bands in 20 samples, with an average of 6–7 loci amplified by each primer. There were not any variations (missing or increasing) of these loci among 20 regenerated bulblets (Fig. 9), indicating that the germplasm maintained a good genetic stability after ultra-low temperature preservation.

4. Discussions

The limitations in the preservation of endemic plant species are variable because of their high diversity, geographic range, and/or accessibility. Research on this matter is urgent and the potential for future scientific studies on the cryopreservation of endemic plant species is enormous. Several factors and their interactions influence the success of any cryopreservation protocol. Understanding these factors, such as the length of dormant buds, preculture, plant physiology and dehydration period, will contribute to a faster and easier optimization of new cryopreservation procedures (Charoensub et al. 2003; Chen et al. 2011; Lambardi et al. 2000; Niino et al. 1992). In general, the small material can be seriously damaged in dehydration process which is difficult to regenerate; however, if the material is too large, the cryoprotectant solution is difficult to infiltrate into the interior cells, the material may not be protected well in the following freezing step, and the regeneration rate is also low. It is found that the optimal shoot-tip size of sweet potato [*Ipomoea batatas* (L.) Lam.] was 0.5-1.0 mm long (Pennycooke and Towill 2000). The survival rate of 1.5-3.0 mm long garlic shoot apices after cryopreservation was as high as 85%, and the regeneration rate was more than 73% (Baek et al. 2003). Wang et al. used in vitro-grown shoot tips of *Vitis vinifera* L. as experimental materials and found that the highest survival (about 65%) of cryopreserved shoot tips was obtained in 1.0-1.5 mm length (Wang et al. 2003). In our study, high regeneration rate was obtained using 2–5 mm long dormant buds. Therefore, the optimal size of dormant buds need to be investigated depending on material and protocol for cryopreservation.

Materials pretreated can effectively reduce the water content of tissues, and enhance the resistance to low temperature, so that the materials can maintain high viability after cryopreservation (Salaj et al. 2010; Hong and Yin 2012; Sershen et al. 2012). Sucrose preculture and cold acclimation are the two most commonly used methods for improving plant recovery in cryopreservation. In many cryopreservation documents, such as *Vitis* (Volk et al. 2018), kiwifruit (Mathew et al. 2018), potato (Kaczmarczyk et al. 2008), *Pyrus* cordata (Chang and Reed 2001), *Prunus cerasus* (Barraco et al. 2012), etc all obtained good results using high concentration of sucrose preculture. In this study, both survival rate and regeneration rate of the dormant buds of *F. przewalskii* reached the highest (93%) when the buds were precultured on

0.5 M sucrose for 3 days. It indicated that high concentration of sucrose in the medium could induce cell dehydration by increasing osmotic pressure, thus reduced the content of free water in cells and improved its cold resistance (Bettoni et al. 2021; Chen et al. 2014; Kulus et al. 2018). Many studies have shown that cold acclimation can activate the cold resistance mechanism in plants and improve the survival of dormant buds in cryopreservation, especially for low-temperature sensitive plants (Channuntapipat et al. 2000; Engelmann 2011; Harding et al. 2009). For example, with cold hardening 6–14 weeks, the regrowth rate of the dormant buds of *Malus domestica* Borkh. had a maximum of 88% regrowth in the 6th and 8th week and were still capable of regenerating at 14 weeks (Bilavčík et al. 2012). The survival rate of blueberry was highest (91%) when the shoot tips were treated at low temperature for 2 to 4 weeks (Wang et al. 2017). In present study, both survival and regeneration rates of the dormant buds of *F. przewalskii* after cryopreservation were increased with the extension of time of cold acclimation.

Vitrification solution makes intracellular water too late to form ice crystals or ice crystals do not have enough time to grow, thus the cells turn a state of artificial complete vitrification (Sakai et al. 1990; Volk and Walters 2006; Rall 1987). At present, the most commonly used vitrification solution is PVS2, and the optimal dehydration time of PVS2 is varied among different plant species (Bilavčík 2012; Wu et al. 2006; Matsumoto et al. 2015; Sant et al. 2006). If the PVS2 treatment time is insufficient, the cell dehydration is not complete, and the cells are easy to form ice crystals when frozen in liquid nitrogen, and the cells will be seriously injured. On the other hand, if the treatment time is too long, the material can be poisoned by PVS2, the survival rate and regeneration rate will significantly reduce (Matsumoto et al. 2015; Wang et al. 2014). Therefore, the appropriate dehydration time in PVS2 can reduce cell damage and improve cell tolerance to freezing. The results of this experiment showed that both PVS2 and PVS3 were good vitrification solution.

Unloading is a normal step in cryopreservation process. But in our study unloading had little effect on the survival and regeneration of the dormant buds after cryopreservation, which is in accordance with cryopreservation for dormant buds of *Codonopsis pilosula* (Franch.) Nannf. (Zhang et al. 2020) and *Astragalus membranaceus* (Fisch) Bge. Var. *Mongholicus* (Bge) Hsiao (Dong et al. 2022). It indicated that dormant bud possessed strong resistance to cryopreservation.

The alternation and damage of dormant buds in process of cryopreservation can be observed by means of histological section. After dehydration of the dormant buds with the loading solution, the cells in each part lost water and showed slight plasmolysis. The cells were subjected to higher osmotic pressure and dehydrated severely by PVS2 treatment, which resulted in further plasmolysis (Popov et al. 2004). Cryopreserved cells were subjected to mechanical damage at the same time, presenting severe plasmolysis, cell membrane rupture, nuclear fragmentation and nucleolus disappearance. After direct cryopreservation of dormant buds, the cells in other parts were irreversibly damaged except for the apical meristem and leaf primordium, which still maintained integrity. The occurrence of cell damage is likely due to the extreme decline in water content, resulting in the inability of protoplasts to contract and concentrate cell fluid (Ganino et al. 2012). In addition, the freezing damage that causes cell death is caused by intracellular water crystallization during freezing or thawing. Therefore, the freezing and

thawing process had severe damage to the buds, mainly manifested as the breakage of the cell wall of the bud axis, the blurring of the nuclear boundary, the ablation of the cytoplasm, and the disappearance of the nucleolus. Shoot apical meristem and leaf primordium are the least damaged parts, although there has slight plasmolysis in these cells, but the cell structure still maintains integrity. These two parts are the key parts of survival. Similar phenomena were also observed in the cryopreservation of apple (Feng et al. 2013), *Rubus* (Chang and Reed 1999), *Dendrobium* (Antony et al. 2011; Ching et al. 2012; Poobathy et al. 2013), *Brassidium* (Mubbarakh et al. 2014) and other plants.

The genetic stability of cryo-derived plants is the prerequisite that the technique applied to plant germplasm conservation. In our study, dormant buds directly produce bulblets without producing callus, so the risk of genetic variation is low. We did not found any phenotypic and DNA alteration in cryo-regenerated plantlets of *F. przewalskii*. Similarly, genetic stability has also confirmed in the shoot tips or dormant buds after cryopreservation in 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), grape and kiwi (Zhai et al. 2003). The genetic fidelity of regenerated plants ensures the established vitrification-cryopreservation protocol as a reliable technique for long term storage of *F. przewalskii* germplasm.

Conclusion

This study showed the possibility of using cryopreservation for the long-term storage of *F. przewalskii*. Notably, a great advantage of this method is the ability to collect material for freezing directly from in situ plant and dormant buds is a superior material for cryopreservtion. This study found that the survival and regeneration rate of dormant buds of *F. przewalskii* were as high as 93% after cryopreservation. Besides cryo-derived plantlets maintained its genetic stability. Histological studies showed that the loading solution and glass protective solution had little effect on tissue cells during cryopreservation, and the effect on liquid nitrogen freezing and defreezing was greater. The use of PVS2 protective agent at the appropriate time could reduce the formation of ice crystals during liquid nitrogen freezing, thus reducing the damage. By comparison, it was found that 1–5 layers of leaf primordial cells and apical meristem cells were the main survival sites.

This approach will provide theory and technology support for establishment cryo-banks of *F. przewalskii* germplasm, meanwhile the research may increase the interest in endemic plants and encourage the implementation of dormant bud cryopreservation.

Declarations

Author contribution

Yuming He conducted experiments, analyzed data, and wrote the manuscript. Huan Sun wrote, and revised the manuscript. Hui Fan analyzed data. Chunyu He, Qingyi Guo and Yanhong Zhang conceived

and designed research. Yanhong Zhang conceived and designed research and revised the manuscript.

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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.

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Figures

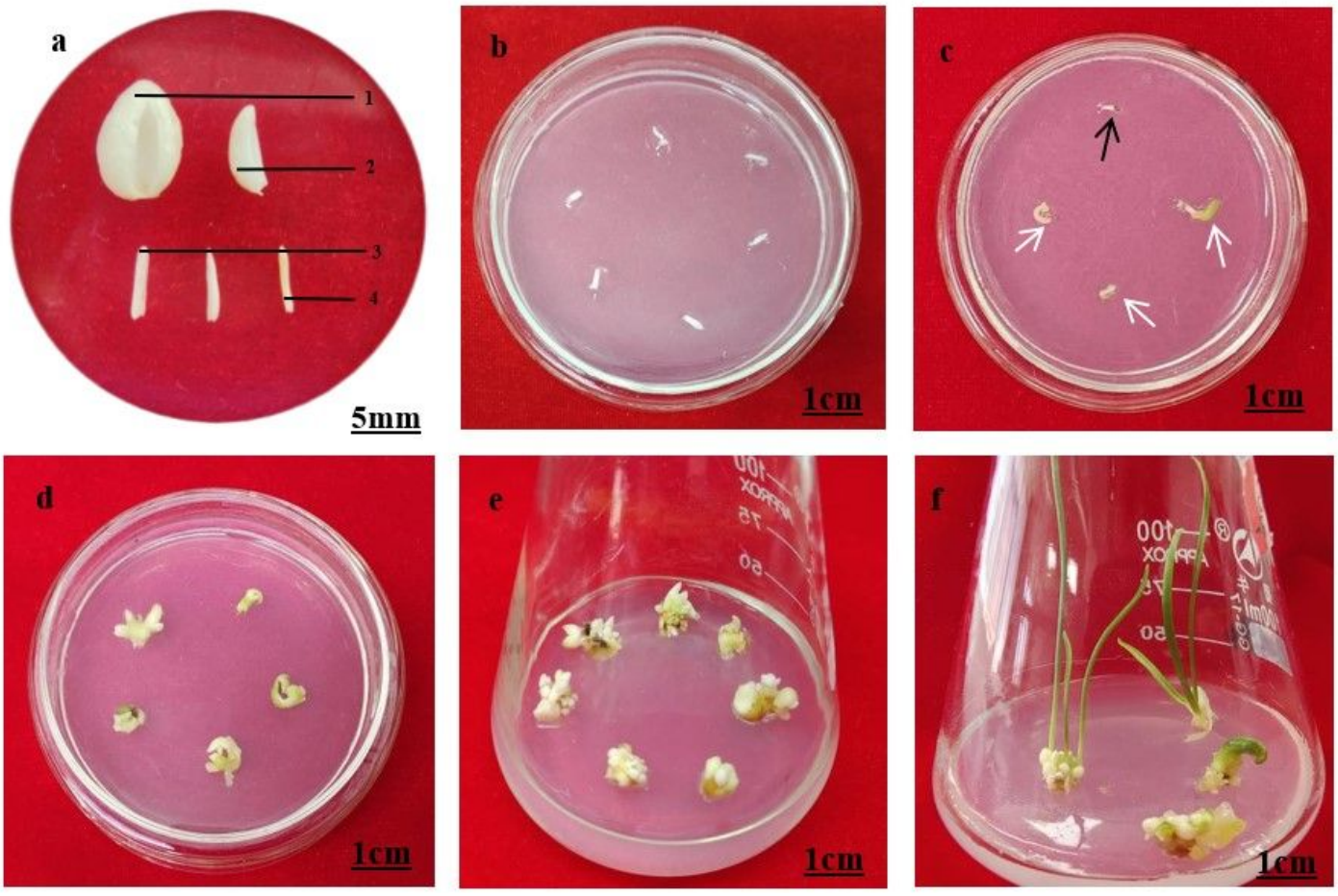


Figure 1

The anatomical structure of bulbs of *Fritillaria przewalskii* and the recovery process of dormant buds after cryopreservation. a Bulb morphology (1, Large scale petal; 2, Small scale petal; 3, Small scale leaf; 4, Dormant buds). **b** Dormant buds after cryopreservation. **c** Recovery of dormant buds cultured for 7 days (white arrows indicate viable dormant buds, black arrows indicate dead dormant buds). **d** Dormant buds produce small bulblets. **e** Subculture propagation of small bulblets. **f** Regenerated seedlings from bulblets.

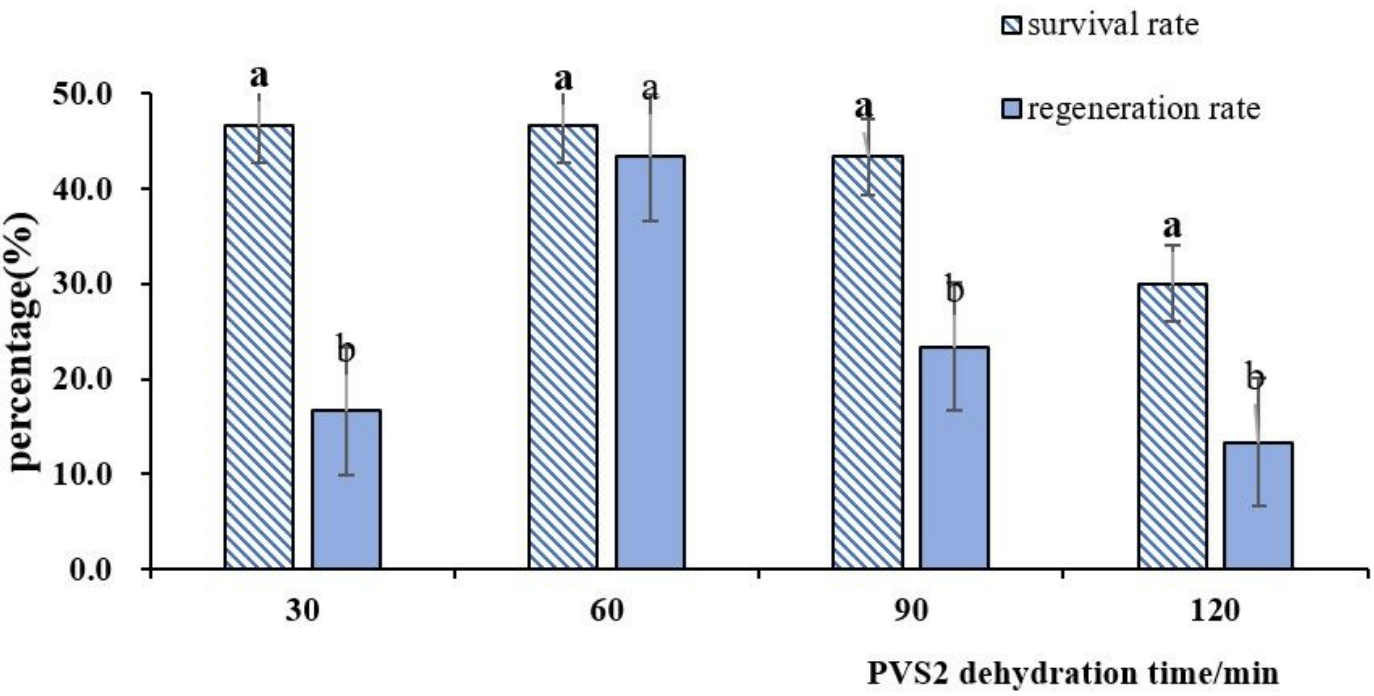


Figure 2

Effect of PVS2 dehydration time on survival rate and regeneration rate of dormant buds after cryopreservation.

Different letters are significantly different from each other at $P < 0.05$, using Duncan's Multiple Range Test. The same as below.

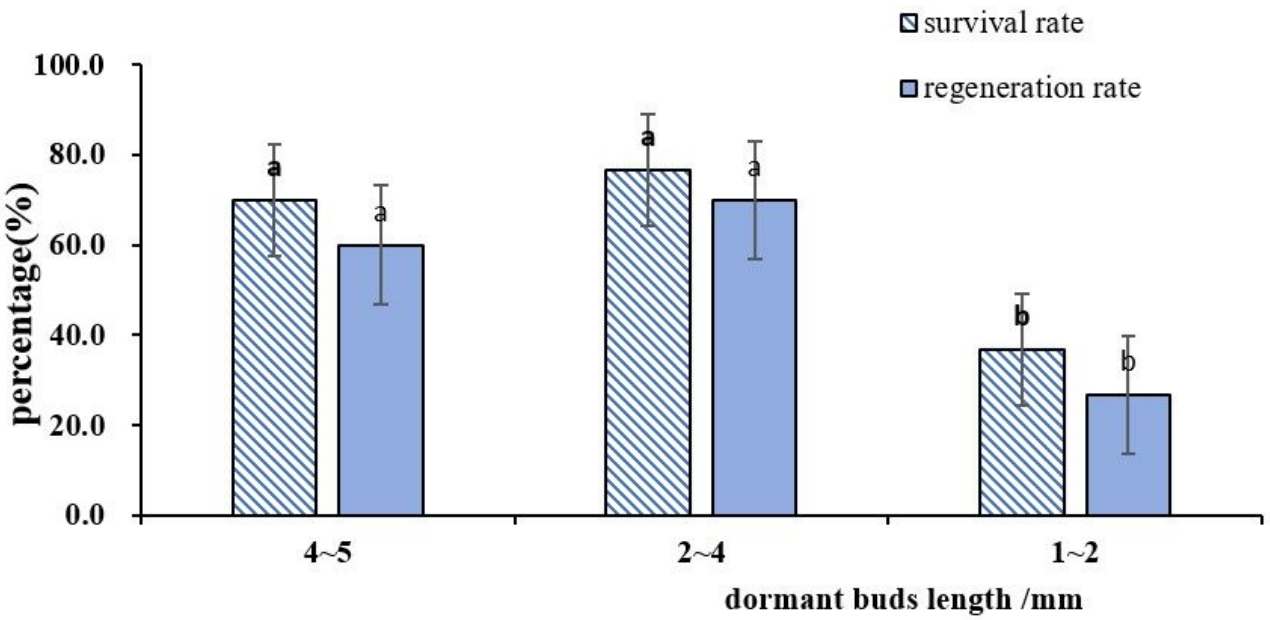


Figure 3

Effect of size (length) of dormant buds on survival rate and regeneration rate after cryopreservation.

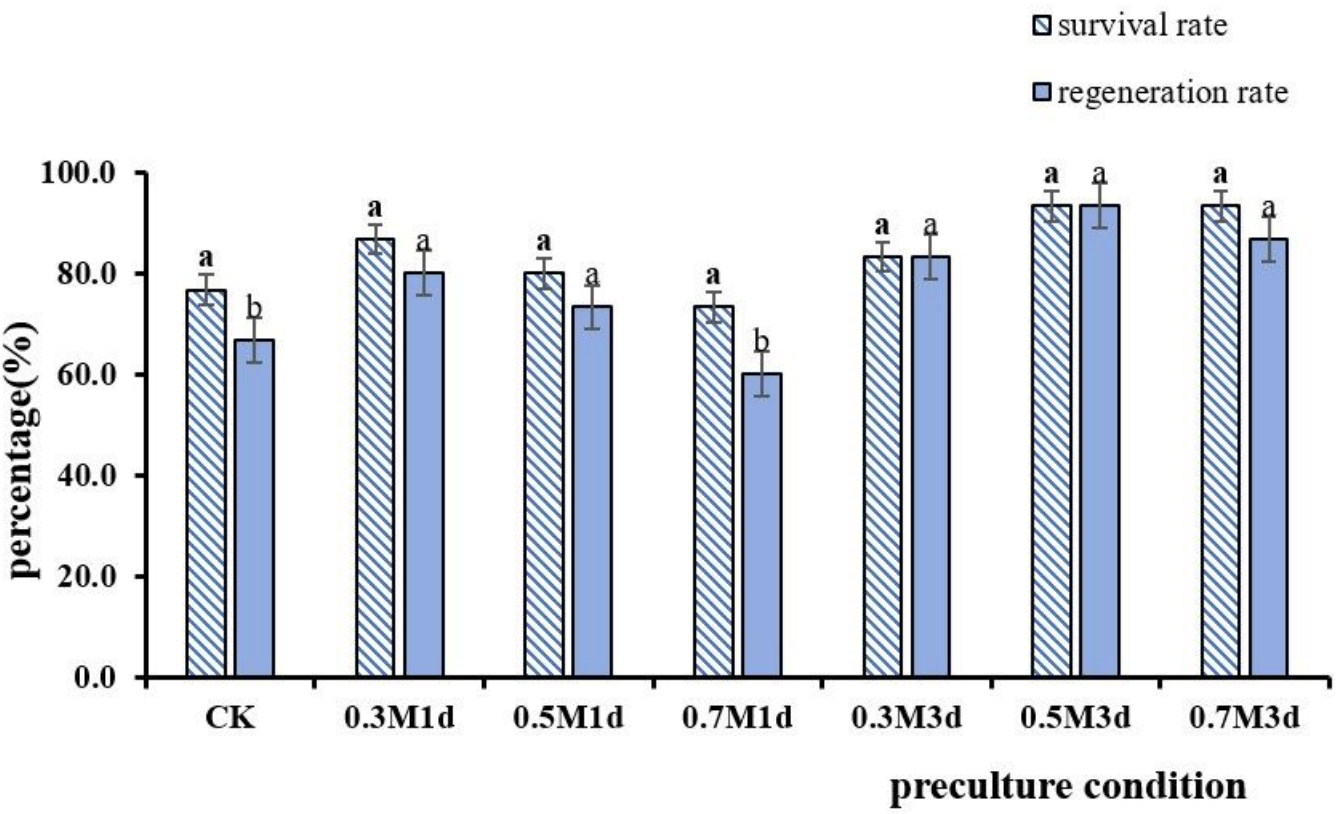


Figure 4

Effects of preculture sucrose concentration and treatment time on survival rate and regeneration rate of dormant buds after cryopreservation.

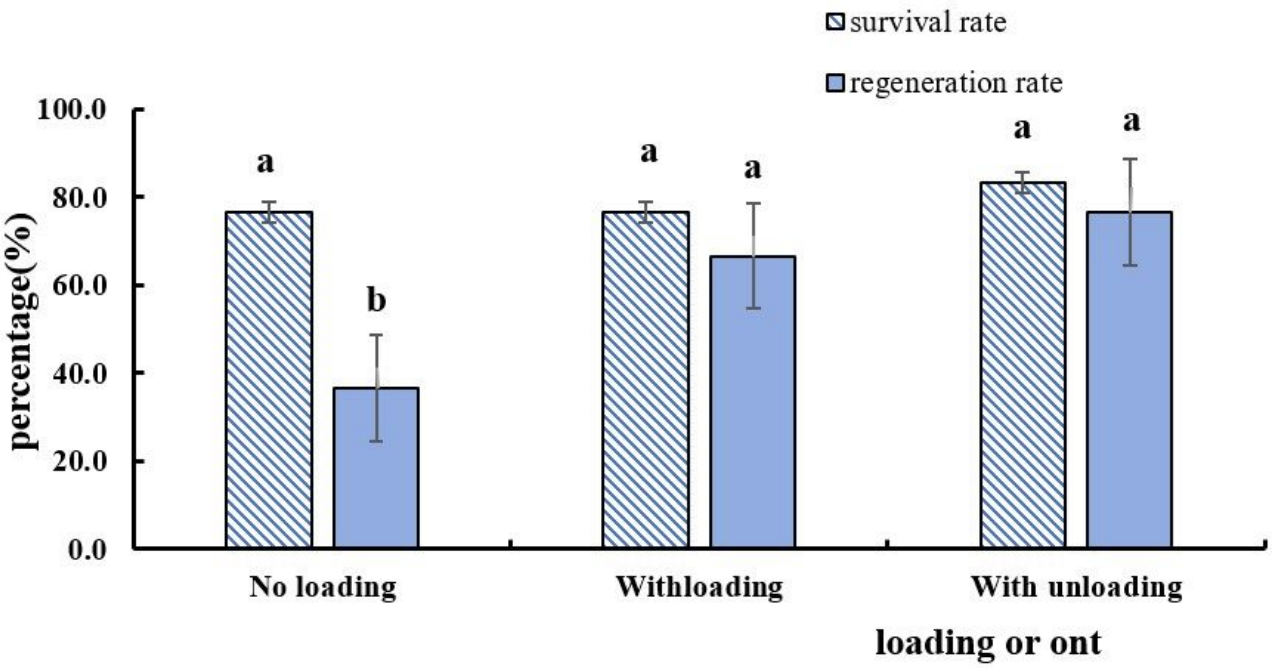


Figure 5

Effects of loading and unloading on survival rate and regeneration rate of dormant buds after cryopreservation.

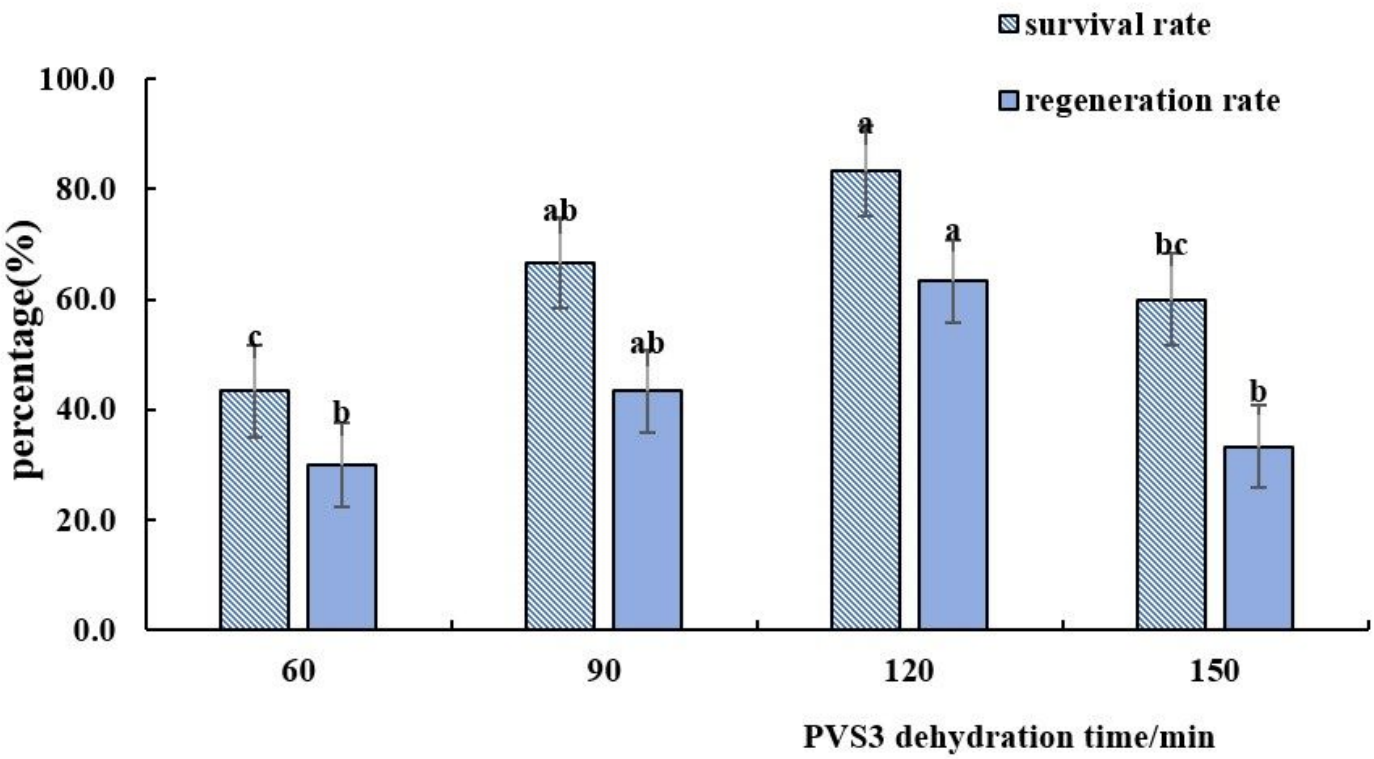


Figure 6

Effect of PVS3 dehydration time on survival rate and regeneration rate of dormant buds after cryopreservation.

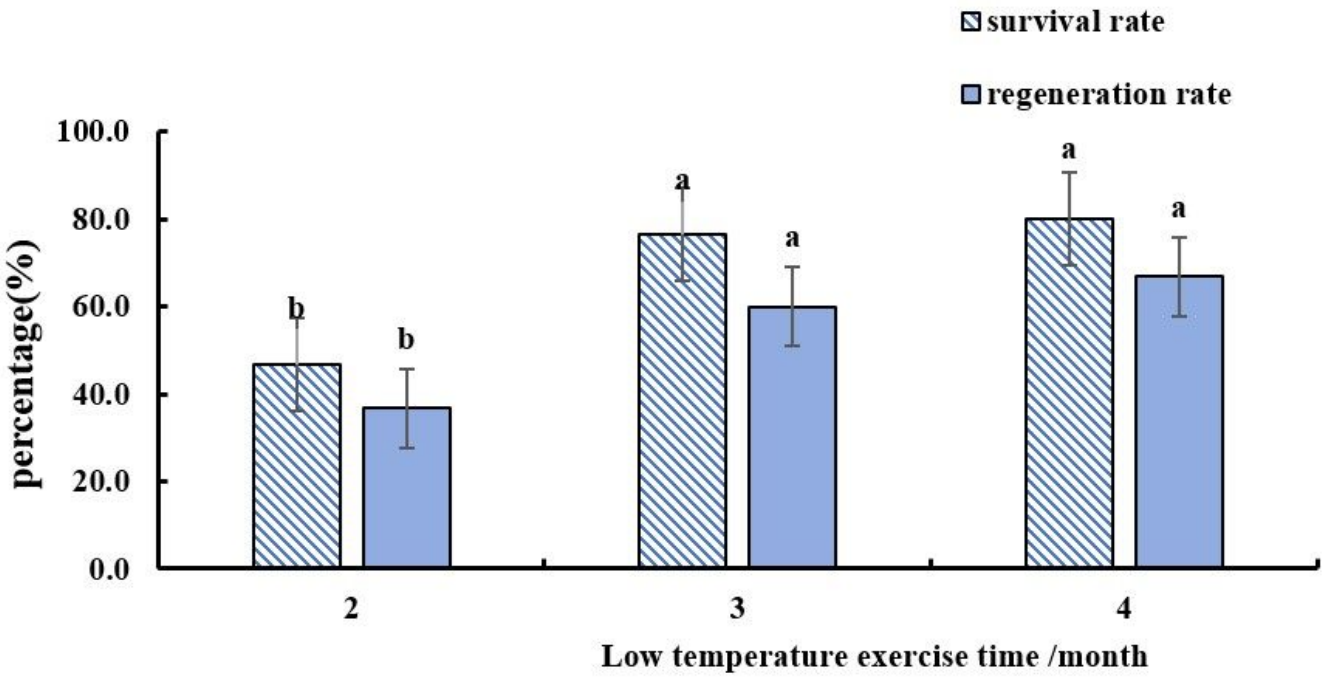


Figure 7

Effects of low temperature exercise time on survival rate and regeneration rate of dormant buds after cryopreservation.

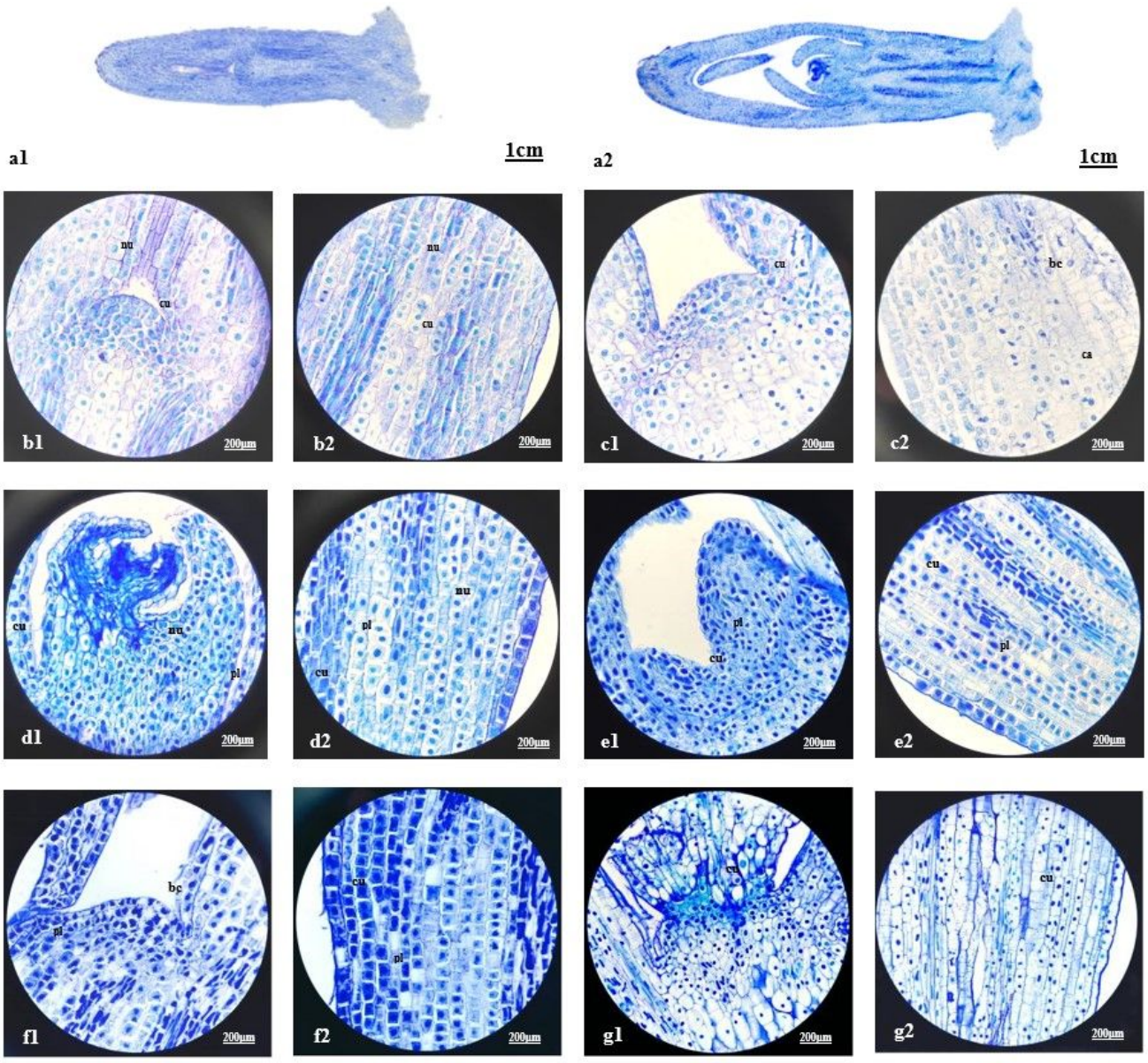


Figure 8

Histological changes of dormant buds of *F. przewalskii* in the process of cyroprervation for the tissue slices from paraffin sectioning and toluidine blue staining under microscope. a1 and a2 represent the complete picture of dormant buds at 4x; b1-g2 represents the CK+, CK-, LS, P_{60}, LN_{60}, and R_{60} leaf primordia and bud axis cells at 40x, respectively; abbreviation **cu,** cell nucleus; **nu,** nucleolus; **pl,** plasmolysis; **bc,** broken cells; and **ca,** cell cavity.

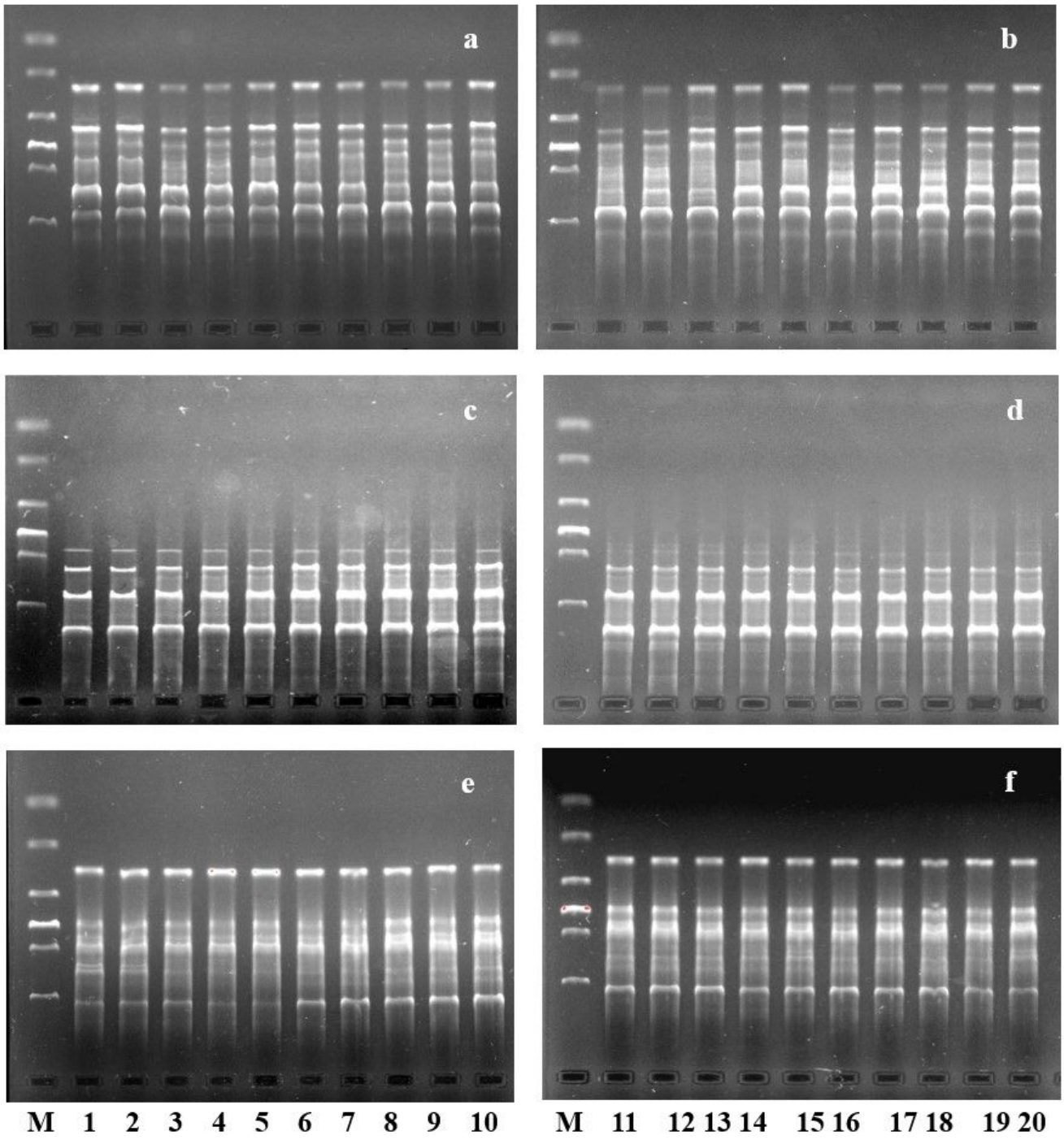


Figure 9

The RAPD genotypic profiles for 20 regenrated plants from dormant buds of *F. przewalskii* after cryopreservation. Image a and b is the amplification profiles from primer s303, c and d from primer s308, and e and f from primer s28.