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Cryopreservation of *Fraxinus mandshurica* Rupr. by Using the Slow Cooling Method

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Abstract: Cryopreservation is an important method for the excellent long-term preservation of plant germplasm. This study explores an optimal cryopreservation technology for the embryogenic callus of *Fraxinus mandshurica* to effectively maintain its genetic stability and morphogenesis potential. The optimal cryopreservation conditions were assessed using the embryogenic callus of *F. mandshurica* as the material, and the slow cooling method was optimized for its cryopreservation. The results indicated that the preculture of embryogenic callus in 0.4 mol·L^{-1} sorbitol solution for 20 h at room temperature, followed by its cryoprotection in 7.5% dimethyl sulfoxide solution at 0 °C for 90 min, constituted the optimal material treatment method. The freezing tube was placed in a −80 °C refrigerator for 2 h and then quickly put into liquid nitrogen for frozen storage. During thawing, the cryopreservation tube was taken out from liquid nitrogen, directly placed in a water bath at 40 °C for 2 min, and used for culturing on the woody plant media + 0.1 mg·L^{-1} 6-benzyladenine + 0.15 mg·L^{-1} 2, 4-dichlorophenoxyacetic acid. After cryopreservation using the slow cooling method, the highest survival rate of callus cells was 80.82%. The fresh weight reached 1.93 g after 60-day recovery culture. The regeneration rate and the proliferation coefficient of the callus were 100% and 2.79, respectively. The differentiation rate was 56.83%, and the emergence rate was 23.59%. The results provide a scientific basis for the long-term preservation of *F. mandshurica* germplasm resources.

Keywords: *Fraxinus mandshurica*; cryopreservation; slow cooling; embryogenic callus; regeneration

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1. Introduction

Plant germplasm resources constitute the basis for maintaining biodiversity and serve as the links between the national economy and ecological lifeline [1]. Because of the extensive application of biotechnology in daily human production practice, people have become increasingly dependent on plant germplasm resources. However, the plant germplasm resources are gradually narrowed in terms of development and usage, and some wild species resources are becoming extinct [2]. In vitro culture of embryogenic callus may cause somatic mutation or a decline or even complete loss of somatic embryogenic potential due to long-term subculturing, especially in conifer species [3,4], which seriously affects the stability of the somatic embryo system and its later usage [5]. Therefore, the

cryopreservation of somatic embryos with liquid nitrogen is important in order to maintain their somatic embryogenic potential.

There are usually two ways of cryopreservation, namely the slow cooling method for cryopreservation and vitrification cryopreservation, and they need to be selected according to different plant materials. Studies have shown that the vitrification cryopreservation cannot effectively preserve the callus of *Fraxinus excelsior*, resulting in a low survival rate. However, the slow cooling method for cryopreservation greatly improves the survival rate [6]. In the slow cooling method for cryopreservation, the samples are pre grown, cryoprotected, slowly cooled (0.5–2.0 °C/min) to a defined pre freezing temperature (typically around −40 °C), quickly immersed in liquid nitrogen for storage, and rapidly thawed and restored [7]. The principle of the slow cooling method is to allow the free water to flow out of the cells by strictly controlling the cooling rate during the freezing temperature storage process to avoid the formation of ice crystals in the cells, which would otherwise cause cell damage [8]. The commonly used preservation materials for slow cooling cryopreservation mainly include suspended cells, callus, tissues, and organs. The physiological state, cold resistance, and genotype of different types of materials reflect different degrees of cryopreservation [9,10]. Thus, the cryopreservation methods suitable for each type of material need to be explored. At present, different types of materials of various conifer species have been successfully preserved and used in the cryopreservation method, leading to enhanced cell activity, growth state, and regeneration rate after recovery.

In a cryopreservation study of *Liriodendron tulipifera*, the suspended cells were incubated in the dark with 0.4 mol·L^{-1} sorbitol for 24 h and further cryoprotected with 5% dimethyl sulfoxide (DMSO). After cryopreservation, survival of the suspended cells reached 100% [11]. The embryogenic callus of *Larix gmelini*, *Pinus elliottii*, and *P. massoniana* could achieve high cell survival and cell regeneration rate under the appropriate cryopreservation method, where the cell regeneration rate of *P. elliotti* and *P. massoniana* was as high as 100% [5,12,13]. The storage conditions of different materials vary greatly, and the most suitable method should be selected according to the characteristics of the materials. However, cryopreservation is currently a highly effective method for maintaining cell survival and preventing variations.

In addition, cryopreservation can effectively maintain genotypic and genetic stability, to ensuring the recovery rate. Somatic embryos of *Picea abies* were pretreated on a semi-solid medium with a high sucrose concentration by using a polyethylene glycol-glucose DMSO (PGD) mixture as the cryoprotectant. Embryo productivity was tested after recovery. An average of 87% of genotypes could be recovered using this method without any effect on their genetic stability [14]. Moreover, it is suggested that the length of telomere seriously affects the quantity and quality of somatic embryos. The in vitro culture time process can lead to shortening of the telomeres. Therefore, the cryopreservation of somatic embryos can effectively maintain the telomere length and reduce cell damage [15]. The evaluation of the genetic stability of regenerated plants after cryopreservation could be enhanced by combined molecular markers or more sensitive methods, such as DNA methylation and gene sequencing [16].

Somatic embryogenesis of *F. mandshurica* has the problems of low somatic embryo yield, unsynchronized somatic embryo development, and a high percentage of deformed somatic embryos. Liu et al. [17] improve *F. mandshurica* somatic embryo production by synchronizing somatic embryo development, improving somatic embryo quality, and inducing root formation to obtain complete regenerated plants. During somatic embryogenesis of *F. mandshurica*, programmed cell death occurs in browning explants, but its potential mechanism is still unclear. The results show that hydrogen peroxide and nitric oxide may play a role in signal transduction and play a key role in somatic embryogenesis and programmed cell death, which preliminarily explains this mechanism [18]. In the long-term subculture and proliferation of embryogenic callus, it is inevitable to increase the rate of cell variation and the loss of embryo, which makes it difficult to achieve the purpose of long-term stable preservation. To maintain the genetic stability and morphogenetic potential of the genetic

material of *F. mandshurica*, we need to develop a suitable cryopreservation method. In this study, optimal conditions for the cryopreservation of *F. mandshurica* embryogenic callus by using the slow cooling method were explored and the precultured and cryoprotection conditions optimized to solve the problem of embryogenic loss of this callus, providing a theoretical basis for the long-term conservation of germplasm resources of *F. mandshurica*.

2. Materials and Methods

2.1. Experimental Materials

The immature (without cryoprotection and with green wings) seeds of *F. mandshurica* were collected from No. 2 open-pollinated mother tree in the Experimental Forest Farm of Northeast Forestry University (126°37′55″ E, 45°43′16″ N) in early August. Cell line 1 of Tree 2 was selected in December 2017 as the successor proliferating cell line. Three embryogenic calluses with different proliferation and differentiation numbers, namely Z2, Z2 (Z3), and Z2 (W2), were cultured in the subculture woody plant media (WPM) + 0.1 mg·L^{-1} 6-benzyladenine (BA) + 0.15 mg·L^{-1} 2,4 dichlorophenoxyacetic acid (2,4 D) for 2 years [19]. The culture conditions were as follows: temperature = 25 ± 2 °C, and humidity = 60–70% in the dark. Differences in the embryogenic callus of three embryogenic callus are given in Table 1.

Table 1. Differences in the proliferation coefficient and somatic embryo induction rate between *F. mandshurica* embryogenic callus under different states.

Callus State	Proliferation Coefficient	Somatic Embryo Induction Rate (%)
Z2	1.32 ± 0.18 b	47.42 ± 2.41 b
Z2(Z3)	1.29 ± 0.06 b	46.78 ± 6.79 b
Z2(W2)	1.41 ± 0.12 a	51.14 ± 4.21 a

Note: The data are expressed as the mean ± SE. Lowercase letters in the same column represent significant differences at $p < 0.05$.

2.2. Method

2.2.1. Cryopreservation of *F. Mandshurica* Embryonic Callus by the Slow Cooling Method

The single factor test method was used for screening freezing conditions for the slow cooling method. The basic conditions were as follows: preculture in 0.4 mol·L^{-1} sucrose solution for 20 h; cryoprotection in 7.5% DMSO cryoprotectant for 60 min; and thawing at 40 °C in a water bath. The fresh weights of the callus were measured at 7, 14, 21, 30, and 60 days after recovery. Survival of the callus was determined using 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining on day 2 of recovery, and each treatment was repeated three times. The test method is as follows:

(1) Screening of embryonic callus materials: 1.0 g of the embryogenic callus Z2, Z2 (Z3), and Z2 (W2) in good growth condition and loose yellowish color were placed in a sterile, 50-mL triangular flask; 0.4 mol·L^{-1} sucrose solution was added to each sample and cultured for 20 h in the dark in a biological shaking table at 100–125 rpm at 25 °C. Subsequently, 7.5% DMSO solution was added to the liquid medium containing the sucrose solution. The flasks containing the callus mixture and the ice–water mixture were collocated for 60 min and the flasks were shaken four times at the same time. After precooling, the DMSO containing callus mixture was transferred to sterile, 1.8-mL freezing tubes, placed in the gradient cooling box precooled in a refrigerator at 4 °C after the lid was closed, and stored in a refrigerator at −80 °C for 2 h. The tubes were immediately immersed in liquid nitrogen, taken out, and thawed in a water bath at 40 °C after 24 h. After thawing, the mixed solution was uniformly dispersed on filter paper in a sterile workbench with a pipette to remove excess water. The sample was then transferred to the basic proliferation medium. The new solid medium was replaced the next day (with DMSO solution completely removed) and cultured in the dark at 25 °C to screen the optimal callus material of *F. mandshurica*.

(2) Concentration of preculture solution: 1.0 g of loose, pale yellow embryonic callus selected from (1) with good growth condition was placed in a sterile, 50-mL triangular flask and cultured on a biological shaking table at 25 °C in the dark at 100–125 rpm for 20 h. The effects of various concentrations of sucrose or sorbitol on the fresh weight and relative survival rate of callus before and after cryopreservation were compared.

(3) Kinds of preculture medium: 1.0 g of the screened loose, pale yellow embryogenic callus with good growth condition was selected and placed in a sterile, 50-mL triangular flask, to which the best selected from (2) of sucrose or sorbitol solutions were added. The callus was then cultured in the dark for 20 h on a biological shaking table at 25 °C and 100–125 rpm. The effects of the two preculture media on the fresh weight and relative survival rate of the callus before and after cryopreservation were compared.

(4) Preculture time: 1.0 g of the screened loose, pale yellow embryogenic callus with good growth condition was placed in a sterile, 50-mL triangular flask, and the best conditions from (3) sorbitol solution was added. The loose, pale yellow embryogenic callus (1.0 g) was cultured in the dark on a biological shaking table at 25 °C at 100–125 rpm for 16, 18, 20, 22, and 24 h to compare the effects of different preculture times on the fresh weight and relative survival rate of the callus before and after frozen storage.

(5) DMSO concentration: 1.0 g of the screened loose, pale yellow embryonic callus in good growth condition was placed in a sterile, 50-mL triangular flask, to which 0.4 mol·L^{-1} sorbitol solution was added. The callus was then cultured in the dark on a biological shaking table at 25 °C and 100–125 rpm for 20 h, followed by cryoprotection in an ice–water mixture for 60 min with the addition of 5%, 7.5%, 10%, and 12.5% DMSO solutions to compare the effects of different DMSO concentrations on the fresh weight and relative survival rate of the callus before and after cryopreservation.

(6) Cryoprotection time: 1.0 g of the screened loose, pale yellow embryonic callus with good growth condition was placed in a sterile, 50-mL triangular flask, to which 0.4 mol·L^{-1} sorbitol solution was added. After culturing for 20 h in the dark on a biological shaking table at 25 °C at 100–125 rpm, 7.5% DMSO solution was added to the liquid medium containing the sorbitol solution, and the samples were cryoprotected in an ice–water mixture for 30, 60, 90, and 120 min. The effects of different cryoprotection time on the fresh weight and relative survival rate of callus before and after cryopreservation were compared.

(7) After the slow cooling cryopreservation and recovery, the embryonic callus of *F. mandshurica* was cultured on WPM + 0.1 mg·L^{-1} BA + 0.15 mg·L^{-1} 2,4-D for regeneration and then subcultured for 15–20 days. Somatic embryo differentiation culture was conducted on Murashige and Skoog (MS) medium ½ + 1.0 mg·L^{-1} BA. Somatic embryo development maturation culture was conducted on MS½ + 1.0 mg·L^{-1} Abscisic acid (ABA) [20]. The emblings were cultured on MS + 0.01 mg·L^{-1} 1-naphthlcetic acid (NAA) for germination and rooting and then photographed for observations.

2.2.2. Determination of Cell Survival and Observation of Recovery Culture before and after Cryopreservation

Fresh weight of callus: The fresh weight of callus was measured on day 0, 7, 14, 21, 30, and 60 after thawing from freezing to observe and record the recovery and growth of callus.

TTC staining method: In total, 100 mg of embryonic callus was placed in a 10-mL centrifuge tube, to which 5-mL of 0.2% TTC solution was added for staining in the dark for 24 h. After the TTC staining solution was removed, the callus was rinsed 5 times with sterile water; 5-mL of 95% ethanol was added to the tube, which was placed in a water bath at 60 °C for 30 min and then cooled to obtain the supernatant. The absorbance was read at 485 nm on an ultraviolet spectrophotometer. The embryonic callus of *F. mandshurica* not preserved at freezing temperature was considered as the control. The relative viability of

cells after cryopreservation for each treatment is expressed as the ratio of absorbance. The calculation formula is as follows:

$$\text{Cell viability} = \frac{\text{Optical Density value of freezing treatment}}{\text{Unprocessed Optical Density value}} \times 100\% \quad (1)$$

Recovery culture observation and recording method: The conditions of callus proliferation, differentiation, and germination during the process of recovery culture of embryonic callus of *F. mandshurica* were observed and recorded regularly. The calculation formulas are as follows:

$$\text{Callus proliferation coefficient} = \frac{\text{Harvest fresh weight}}{\text{Inoculated fresh weight}} \times 100\% \quad (2)$$

$$\text{Callus differentiation rate} = \frac{\text{Number of differentiated callus}}{\text{Total number of inoculated callus}} \times 100\% \quad (3)$$

$$\text{Bud ratio}(\%) = \frac{\text{Number of embryogenic callus budding}}{\text{Total number of embryogenic callus at inoculation time}} \times 100\% \quad (4)$$

2.2.3. Data Statistics and Analysis

All the above experiments were repeated more than three times and data collation was performed using Microsoft Excel 2010, one-way analysis of variance (ANOVA) with SPSS (2015, V. 23, Chicago, IL, USA) and plotted using SigmaPlot (2011, V. 12.5, SYSTAT) software.

3. Results

3.1. Screening of Embryogenic Callus Materials

It was found that Z2 (W2) had a higher Callus proliferation coefficient and somatic embryogenesis induction rate than Z2 and Z2 (Z3) in the preliminary screening of callus in three states (Table 1). The fresh weight of the embryogenic callus of *F. mandshurica* in the three states was increased with an increase in the time of culture. After 60 days of culture, the fresh weight of Z2 (W2) was significantly different from that of the other two calluses ($p < 0.05$) (Table 2). The fresh weights of all three types of callus in the first 14 days of proliferation culture after recovery with the slow cooling method did not change compared with those recorded on day 0 ($p > 0.05$). After 21 days of culture, the fresh weights of the three calluses increased significantly, by 13.13%, 15.46%, and 23.30%, respectively, compared with those on day 0. After 60 days of culture, the fresh weights of the three calluses increased by 47.47%, 83.51%, and 106.80%, respectively, compared with those on day 0. Therefore, Z2 (W2) was considered the optimal embryogenic callus state of *F. mandshurica*.

Table 2. Changes in the fresh weight of *F. mandshurica* embryogenic callus in different states with culture time.

Callus State	Incubation Time (Days)					
	0	7	14	21	30	60
Z2	0.99 ± 0.7 a	0.98 ± 0.6 a	0.99 ± 0.08 a	1.12 ± 0.9 b	1.23 ± 0.08 b	1.46 ± 0.21 b
Z2(Z3)	0.97 ± 0.1 a	1.00 ± 0.2 a	1.01 ± 0.04 a	1.12 ± 0.6 b	1.22 ± 0.10 b	1.78 ±0.21 ab
Z2(W2)	1.03 ± 0.2 a	1.03 ± 0.12 a	1.06 ± 0.08 a	1.27 ± 0.1 a	1.45 ± 0.19 a	2.13 ± 0.19 a

Note: Lowercase letters represent significant differences at $p < 0.05$.

3.2. Effect of the Preculture Conditions on Cell Survival

3.2.1. Effects of Sucrose Concentration on the Fresh Weight of Callus and Cell Survival Rate

Different sucrose concentrations had a significant effect on the fresh weight of the embryonic callus of *F. mandshurica* after recovery from cryopreservation by the slow cooling

method (Table 3). After 14 days of culture, the fresh weight of the callus treated with different sucrose concentrations exhibited no significant change compared with that on day 0. After 21 days of culture, the fresh weight of the callus induced by different sucrose concentrations significantly increased. Under the same sucrose concentration treatment, the fresh weight of the callus increased gradually as the days of culture increased. When the medium was restored to 60 days, the fresh weight of the callus first increased and then decreased with an increase in the sucrose concentration. The fresh weight of the callus with the 0.4 mol·L^{-1} sucrose preculture solution treatment was the highest (1.66 g), which was significantly different (28.68% and 39.50% higher, respectively) from those under 0.3 and 0.7 mol·L^{-1} sucrose preculture solution treatments.

Table 3. Changes in the fresh weight of *F. mandshurica* embryogenic callus with culture time under different sucrose concentrations.

Sucrose Concentration (mol·L^{-1})	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
0.3	0.94 ± 0.4 a	0.93 ± 0.06 c	1.00 ± 0.05 a	1.00 ± 0.03 c	1.13 ± 0.04 b	1.29 ± 0.2 b
0.4	1.01 ± 0.01 a	1.04 ± 0.01 a	1.10 ± 0.03 a	1.25 ± 0.02 a	1.40 ± 0.08 a	1.66 ± 0.21 a
0.5	0.96 ± 0.02 a	1.00 ± 0.02 ab	1.00 ± 0.83 a	1.17 ± 0.09 ab	1.17 ± 0.19 b	1.34 ± 0.05 b
0.6	0.99 ± 0.08 a	0.97 ± 0.08 bc	0.99 ± 0.08 a	1.14 ± 0.08 b	1.16 ± 0.05 b	1.25 ± 0.08 b
0.7	0.95 ± 0.08 a	0.93 ± 0.05 c	0.96 ± 0.06 a	1.13 ± 0.05 b	1.15 ± 0.6 b	1.19 ± 0.06 b

Note: Lowercase letters represent significant differences at $p < 0.05$.

Different concentrations of sucrose in the preculture medium significantly affected cell survival of *F. mandshurica* embryonic callus after cryopreservation and recovery (Figure 1). With an increase in the concentration of sucrose preculture solution, the relative cell viability first increased and then decreased. At a preculture solution concentration of 0.4 mol·L^{-1}, cell viability reached the maximum (64.59%), whereas at a concentration of 0.3 mol·L^{-1}, cell viability was the lowest (38.80%). Cell survival of the callus treated with 0.5, 0.6, and 0.7 mol·L^{-1} of preculture solution was 53.71%, 47.24%, and 44.21%, respectively.

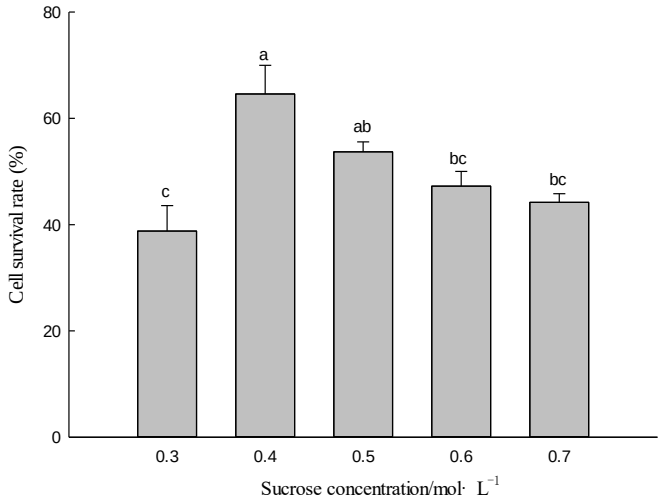

Figure 1. Effect of sucrose concentration on the survival percentage of *F. mandshurica* embryogenic callus after cryopreservation by the slow cooling method. Note: Lowercase letters represent significant differences at $p < 0.05$.

Therefore, the 0.4 mol·L^{-1} concentration of sucrose preculture solution was considered suitable for the cryopreservation of *F. mandshurica* embryogenic callus by using the slow cooling method.

3.2.2. Effect of Sorbitol Concentration on Cell Survival

Different concentrations of sorbitol preculture medium had a significant effect on the fresh weight of *F. mandshurica* embryonic callus after recovery from cryopreservation using the slow cooling method. With an increase in the sorbitol concentration, the fresh weight of the callus in the same culture time tended to increase first and then decrease (Table 4). After subculturing for 60 days, the difference in fresh weight of the callus was extremely significant ($p < 0.05$). The fresh weight of the callus treated with 0.4 mol·L^{-1} sorbitol preculture solution was the highest (1.92 g), which was 34.27% and 54.84% higher than that obtained after treatment with 0.3 and 0.7 mol·L^{-1} sorbitol preculture solutions, respectively. After 21 days of culture, the fresh weight of the callus treated with 0.4 mol·L^{-1} sorbitol preculture solution began to increase significantly compared with that at day 0 of culture. The callus proliferation rate was 120.69%, whereas the callus proliferation rate at the concentration of 0.3, 0.5, and 0.7 mol·L^{-1} was 74.39%, 56.12%, 44.09% and 33.33%.

Table 4. Changes in the fresh weight of *F. mandshurica* embryogenic callus with culture time under different sorbitol concentrations.

Sorbitol Concentration (mol·L^{-1})	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
0.3	0.82 ± 0.03 a	0.86 ± 0.06 a	0.90 ± 0.05 a	0.98 ± 0.04 b	1.13 ± 0.04 b	1.43 ± 0.17 b
0.4	0.87 ± 0.1 a	0.87 ± 0.01 a	0.97 ± 0.02 a	1.23 ± 0.09 a	1.43 ± 0.13 a	1.92 ± 0.15 a
0.5	0.98 ± 0.04 a	0.98 ± 0.02 a	1.00 ± 0.02 a	1.07 ± 0.05 b	1.17 ± 0.05 b	1.53 ± 0.30 b
0.6	0.93 ± 0.07 a	0.96 ± 0.08 a	0.97 ± 0.08 a	1.04 ± 0.01 b	1.13 ± 0.05 b	1.34 ± 0.09 b
0.7	0.93 ± 0.07 a	0.95 ± 0.5 a	0.96 ± 0.06 a	1.01 ± 0.03 b	1.06 ± 0.06 b	1.24 ± 0.03 b

Note: Lowercase letters represent significant differences at $p < 0.05$.

Different concentrations of sorbitol preculture solution have a significant influence on the cell survival rate of *F. mandshurica* embryonic callus after cryopreservation and recovery by the slow cooling method (Figure 2). With the increase of sorbitol concentration, the relative cell survival rate first increased and then decreased. When the sorbitol concentration was 0.4 mol·L^{-1}, the cell survival rate of the recovered callus reached the maximum, which was 80.82%. Compared with 0.3 mol·L^{-1}, the cell survival rate increased by 85.50% respectively. The results showed that the optimum concentration of sorbitol preculture solution for the cryopreservation of embryonic callus of *F. mandshurica* by the slow cooling method was 0.4 mol·L^{-1}.

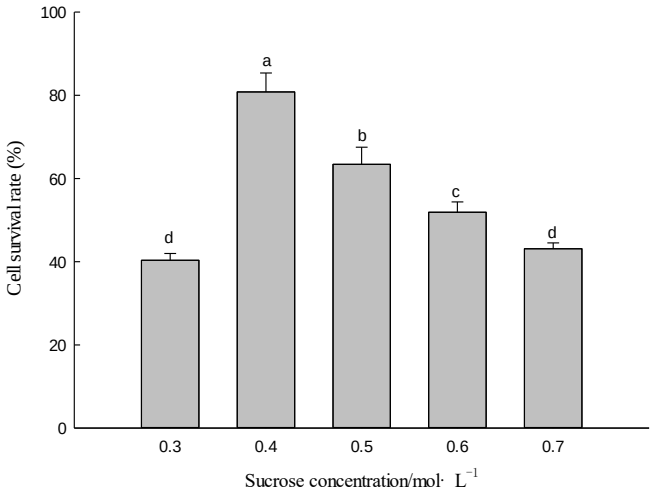

Figure 2. Effect of sorbitol concentration on the survival percentage of embryogenic callus of *F. mandshurica* after cryopreservation by slow cooling. Note: Lowercase letters represent significant differences at $p < 0.05$.

3.2.3. Effects of Different Kinds of Preculture Medium on Callus Fresh Weight and Cell Survival

Under different preculture medium treatments, the fresh weight of embryonic callus of *F. mandshurica* stored by the slow cooling method reached a significant level (Table 5). With the prolongation of culture days, the fresh weight of the callus treated with sorbitol and sucrose preculture solutions increased gradually, and it increased significantly on day 21 of culture. After 60 days of culture, fresh weights of the sorbitol- and sucrose-treated calluses reached the maximum (1.93 g and 1.66 g, respectively) and were 121.84% and 64.36% higher, respectively, than that on day 0. The fresh weight of the callus treated with sorbitol was 16.27% higher than that of the callus treated with sucrose. The two preculture solutions at the same concentration had a significant effect on cell viability ($p < 0.05$). When the preculture solution concentration was 0.4 mol·L^{-1}, cell viability was the highest under both sorbitol and sucrose treatments, although the cell viability under sorbitol treatment was higher than that under sucrose treatment (Figures 1 and 2). The results indicated that the sorbitol solution was the most suitable preculture solution for the cryopreservation of *F. mandshurica* embryonic callus by using the slow cooling method.

Table 5. Effects of the kind of preculture medium on the fresh weight of *F. mandshurica* embryogenic callus after cryopreservation by the slow cooling method.

Type of Preculture Medium	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
Sorbitol (0.4 mol·L^{-1})	0.87 ± 0.15 c	1.01 ± 0.02 c	1.03 ± 0.05 c	1.29 ± 0.02 b	1.43 ± 0.13 b	1.93 ± 0.15 a
Sucrose (0.4 mol·L^{-1})	1.01 ± 0.02 c	1.04 ± 0.01 c	1.05 ± 0.15 c	1.22 ± 0.06 b	1.36 ± 0.05 b	1.66 ± 0.21 a

Note: The lowercase letters represent significant differences at $p < 0.05$.

3.2.4. Effect of Preculture Time on Fresh Weight and Survival Rate of the Callus

During cryopreservation by the slow cooling method, different preculture times had significant effects on the fresh weight and cell survival rate of *F. mandshurica* embryogenic callus. When cultured up to 21 d with the addition of 0.4 mol·L^{-1}, the fresh weight of the callus under each treatment was significantly higher than that at day 0 (Table 6). On the same culture day, with the extension of preculture time, the fresh weight of the callus tended to first increase and then decrease. The fresh weight of the callus with a preculture time of 16 h was the lowest (1.31 g) at day 60. When the preculture time was 20 h, the fresh weight of the callus reached the maximum (1.98 g), which was significantly different from those under other treatments ($p < 0.05$) and 51.15% higher than that with 16 h preculture.

Table 6. Changes in the fresh weight of *F. mandshurica* embryogenic callus with different preculture times.

Time (h)	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
16	0.99 ± 0.15 a	1.00 ± 0.02 a	1.06 ± 0.05 a	1.13 ± 0.04 b	1.21 ± 0.14 c	1.31 ± 0.17 c
18	0.95 ± 0.17 a	0.95 ± 0.06 a	1.02 ± 0.02 a	1.21 ± 0.06 ab	1.37 ± 0.13 b	1.54 ± 0.15 b
20	0.92 ± 0.11 a	0.94 ± 0.06 a	1.02 ± 0.02 a	1.31 ± 0.05 a	1.51 ± 0.05 a	1.98 ± 0.30 a
22	0.99 ± 0.01 a	1.01 ± 0.02 a	1.04 ± 0.18 a	1.13 ± 0.05 b	1.22 ± 0.03 b	1.43 ± 0.1 bc
24	0.95 ± 0.05 a	1.00 ± 0.01 a	1.04 ± 0.06 a	1.14 ± 0.03 b	1.21 ± 0.07 c	1.35 ± 0.05 c

Note: The data are expressed as the mean ± SE. Different lowercase letters in the same column represent significant differences at $p < 0.05$.

With the extension of culture time, the cell survival rate of the recovered callus first increased and then decreased (Figure 3). The cell survival rate was the highest (68.8%) when the preculture time was 20 h, and the difference in the cell survival rate was significant between the treatment with 20-h preculture and those with other preculture times. The

cell survival rates at 16 h, 18 h, 22 h, and 24 h treatment were 24.87%, 49.34%, 43.99%, and 41.38%. The results indicated that the most suitable preculture time for the cryopreservation of *F. mandshurica* embryonic callus by using the slow cooling method was 20 h.

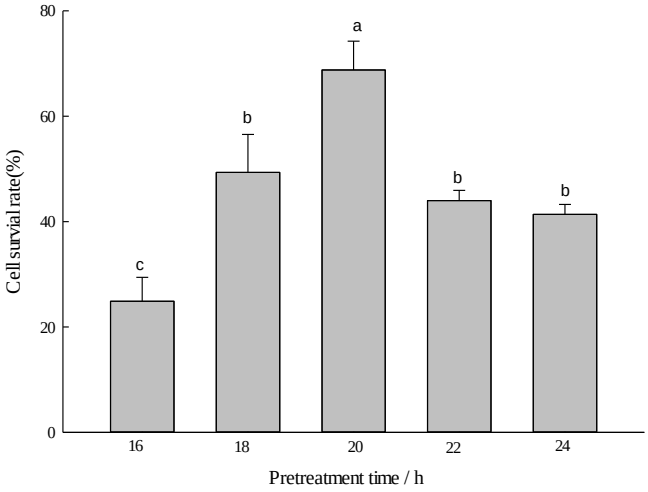

Figure 3. Effect of preculture time on the cell survival percentage of *F. mandshurica* embryonic callus after cryopreservation by the slow cooling method. Note: Lowercase letters represent significant differences at $p < 0.05$.

3.3. Effect of the Cryoprotection Conditions on Cell Survival

3.3.1. Effect of DMSO Concentration on the Fresh Weight and Cell Survival of the Callus

DMSO concentration had significant effects on the fresh weight and cell survival rate of *F. mandshurica* embryogenic callus. After 60 days of culture, the fresh weight of the callus first increased and then decreased with an increase in the DMSO concentration (Table 7). When the DMSO concentration was 7.5%, the fresh weight of the callus was maximum (1.74 g), which was 83.16% higher than that on day 0. The difference in fresh weight was significant compared with those under other treatments ($p < 0.05$). With the DMSO concentrations of 5%, 7.5%, 10.0%, and 12.5%, the cell survival rate of the callus was 34.12%, 82.42%, 65.99%, and 52.40%, respectively (Figure 4). The cell survival rate was the highest when the DMSO concentration was 7.5%. Therefore, the DMSO concentration of 7.5% was deemed the most suitable for the cryopreservation of *F. mandshurica* embryonic callus by using the slow cooling method.

Table 7. Effect of DMSO concentration on the fresh weight of *F. mandshurica* embryogenic callus after cryopreservation by the slow cooling method.

DMSO Concentration (%)	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
5.0	0.99 ± 0.02 a	1.00 ± 0.01 a	1.06 ± 0.04 a	1.13 ± 0.04 b	1.15 ± 0.14 c	1.19 ± 0.03 c
7.5	0.95 ± 0.07 a	0.95 ± 0.06 a	1.03 ± 0.02 a	1.28 ± 0.07 a	1.41 ± 0.13 a	1.74 ± 0.15 a
10.0	0.92 ± 0.11 a	0.94 ± 0.05 a	1.03 ± 0.02 a	1.20 ± 0.07 ab	1.29 ± 0.04 b	1.43 ± 0.30 b
12.5	0.99± 0.01 a	1.01 ± 0.02 a	1.04 ± 0.01 a	1.13 ± 0.05 b	1.2 ± 0.3 bc	1.27 ± 0.6 bc

Note: The data are expressed as the mean ± SE. Different lowercase letters in the same column represent significant differences at $p < 0.05$.

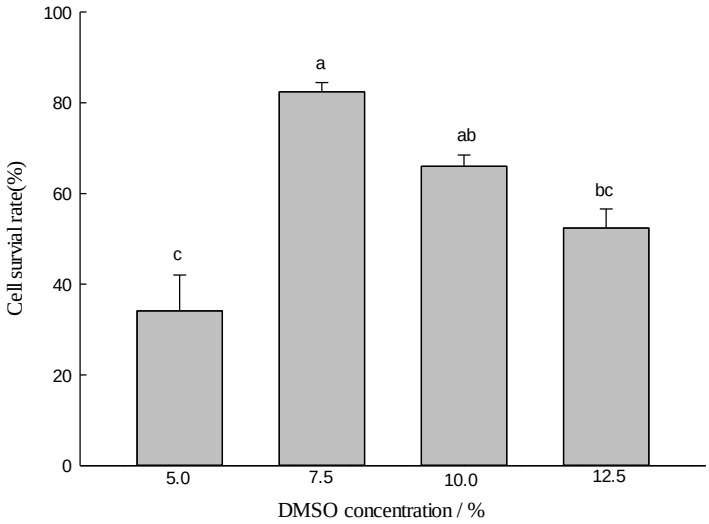

Figure 4. Effect of DMSO concentration on the cell survival percentage of *F. mandshurica* embryonic callus after cryopreservation by the slow cooling method. Note: Lowercase letters represent significant differences at $p < 0.05$.

3.3.2. Effect of Cryoprotection Time on Fresh Weight and Cell Survival Rate of the Callus

During cryopreservation by the slow cooling method, cryoprotection time had a significant effect on callus fresh weight (Table 8). With the extension of culture time, the fresh weight of the callus gradually increased, reaching the maximum on day 60 of culture. When the cryoprotection time was 90 min, the fresh weight of the callus was the highest (1.87 g), which was 88.89% higher than that on day 0 of recovery culture. When the cryoprotection time was 120 min, the fresh weight of the callus was the lowest (1.18 g) and was 19.19% higher than that on day 0. The fresh weight of the callus with cryoprotection times of 90 and 120 min differed significantly (Table 8). With the gradual extension of cryoprotection time, the cell survival rate of the callus exhibited an increasing trend first and then a decreasing trend (Figure 5). When the cryoprotection time was 90 min, the cell survival rate reached the maximum (71.60%), whereas when the cryoprotection time was 120 min, the cell survival rate was the lowest (24.39%). Therefore, the cryoprotection time of 90 min was considered the most suitable for the cryopreservation of *F. mandshurica* embryonic callus by using the slow cooling method.

Table 8. Changes in the fresh weight of *F. mandshurica* embryogenic callus with culture under different cryoprotection times.

Cryoprotection Time (min)	Incubation Time (Days)					
	0	**7**	**14**	**21**	**30**	**60**
30	0.96 ± 0.6 a	0.99 ± 0.01 a	0.99 ± 0.02 a	1.06 ± 0.04 b	1.15 ± 0.3 b	1.28 ± 0.3 b
60	0.91 ± 0.7 a	0.95 ± 0.06 a	0.94 ± 0.05 a	1.08 ± 0.07 b	1.30 ± 0.1 ab	1.61 ± 0.15 a
90	0.92 ± 0.1 a	0.92 ± 0.04 a	0.96 ± 0.08 a	1.25 ± 0.13 a	1.42 ± 0.04 a	1.87 ± 0.30 a
120	0.99 ± 0.2 a	1.01 ± 0.02 a	1.02 ± 0.03 a	1.09 ± 0.05 b	1.1 ± 0.32 b	1.18 ± 0.32 b

Note: The data are expressed as the mean ± SE. Different lowercase letters in the same column represent significant differences at $p < 0.05$.

3.4. Somatic Embryogenesis and Plant Regeneration after Recovery

After cryopreservation by the slow cooling method, the embryonic calluses of *F. mandshurica* were cultured for one day, following which they turned milky and thick (Figure 6a). After subculture and proliferation for 30 days, the callus became pale yellow and loose (Figure 6b), with a proliferation coefficient of 2.79. The proliferation coefficient of the callus without cryopreservation (control) was 2.86. Further, the calluses were transferred to the differentiation medium (Figure 6c). The somatic embryo could be differentiated normally. The

callus differentiation rate was 56.83%, and that of the control was 59.44%. After two months, the calluses were transferred to the maturation medium (Figure 6d), following which the number of somatic embryos increased significantly. After two months, the sprouting and rooting of cultures were conducted, and the somatic embryos could germinate normally to form plantlets (Figure 6e). The emergence rate after freezing temperature treatment was 23.59%, whereas that without freezing temperature treatment was 25.87%. After one month, the calluses were transferred to a can for rooting culture (Figure 6f). In summary, after the *F. mandshurica* embryogenic calluses were cryopreserved by the slow cooling method, the callus proliferation coefficient, callus differentiation rate, and emergence rate decreased but did not differ significantly from those of the control. This finding suggested that the slow cooling method was suitable for the cryopreservation of *F. mandshurica* embryogenic callus.

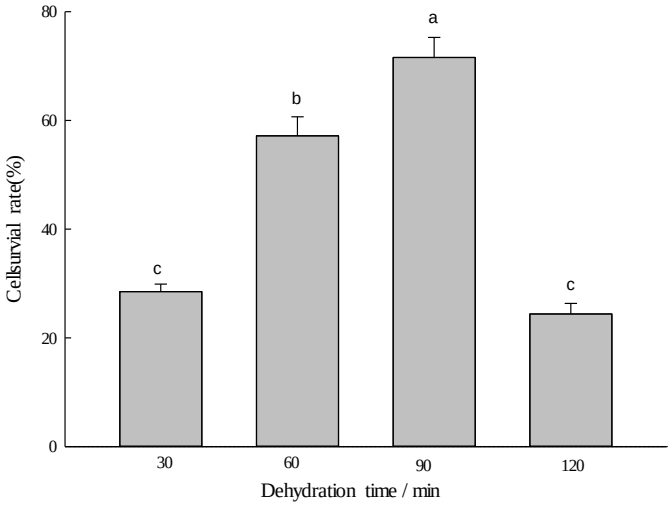

Figure 5. Effect of cryoprotection time on the cell survival percentage of *F. mandshurica* embryonic callus after cryopreservation by the slow cooling method. Note: Lowercase letters represent significant differences at $p < 0.05$.

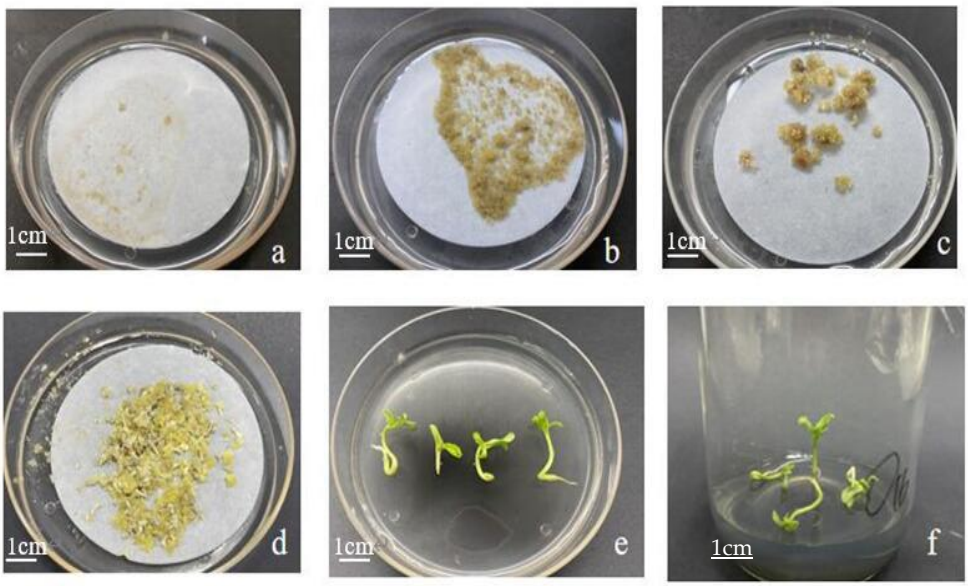

Figure 6. Recovery culture process of embryonic callus of *F. mandshurica* after cryopreservation by the slow cooling method. (**a**,**b**) *F. mandshurica* embryonic callus at day 1 and day 30 of culture; (**c**) somatic embryo callus differentiation; (**d**) somatic embryo maturation culture; (**e**) germination of the somatic embryos of *F. mandshurica* and the appearance of roots; (**f**) *F. mandshurica* emblings.

4. Discussion

Since its development in the 1960s, plant cryopreservation has been regarded as an extraordinary method for the safe long-term preservation of biological materials because it does not induce genetic alterations and maintains the regenerative potential of storage materials [21]. Cryopreservation has been widely used in various fields. Moreover, after cryopreservation, an embryogenic callus can be stored for an indefinite period of time to ensure its cell genetic stability and somatic embryogenesis potential [22]. The tissue is precultured and appropriately cryoprotected before cryopreservation to reduce the water content in the cells, synchronize the cell division and differentiation, and enhance the anti-adversity ability of the material in the case of severe temperature changes and high degree of cryoprotection during cryopreservation. The type of preculture solution, preculture time, and the type and concentration of cryoprotectant affect the slow cooling cryopreservation process and are important factors for cell survival [14,23].

4.1. Effect of Preculture Condition on Fresh Weight and Cell Survival Rate of Callus

In the preculture of conifer embryogenic callus, to reduce cell damage, the osmotic pressure is usually kept high by increasing the concentration of the osmotic agent so that the cells are gradually cryoprotected and the cold resistance of cells is increased. Embryonic callus of *P. elliottii* was precultured for 12, 24, 36, and 48 h in a hypertonic medium with 0.5 mol·L^{-1} sucrose. After 12 h of preculture, cell viability after thawing was low because of freezing injury caused by a high free water content in cells. However, after 48 h, cell viability decreased significantly, and 24 and 36 h were the best times for the cryopreservation and culture of *P. elliottii* callus. After thawing in a 32 °C water bath, it resumed growth after one month, and the callus regeneration rate reached 100%. The callus of *P. koraiensis* was pretreated for 0, 12, 24, 36, and 48 h, and the relative cell survival rate was found to be the highest when the pretreatment time was 24 h [24]. The finding is consistent with our finding indicating that 20 h is the optimal preculture time, which further suggests that the appropriate preculture time is beneficial to the recovery of cell growth in the later period (Table 6).

Studies on cryopreservation of *P. massoniana* embryonic callus have reported the effect of callus recovery from pretreatment for 36–48 h rather than 12–24 h. Long-term pretreatment reduces the water content of cells and is considered beneficial to maintain cell viability [12]. Generally, it is considered that the lower the water content of callus, the higher the cryopreservation efficiency. However, Chen et al. [25] studied the callus of *Oryza sativa* and reported that the low water content of the callus did not necessarily improve the cryopreservation efficiency. As a result of osmotic pressure, cells lose more water, resulting in a high concentration of cytoplasm. This destroys the ion balance in cells and reduces the physiological activity of cells, thus leading to cell proliferation. Therefore, for different materials, the optimal preculture time should be determined by combining the recovery growth rate and time required for recovery growth.

Sucrose and sorbitol solutions are widely used as common penetrants in production practice. In this study, the cell viability of the *F. mandshurica* embryonic callus precultured in 0.4 mol·L^{-1} sorbitol solution for 21 h was up to 80.82%, indicating that sorbitol solution was more suitable for the preculture of *F. mandshurica* embryonic callus (Figure 2). In addition to the traditional sucrose or sorbitol solutions as the basic osmotic agent, *Picea omorika* could effectively improve the recovery rate when it was used for the pretreatment of embryogenic cultures on a semi-solid medium with a high sucrose concentration and a PGD mixture as the cryoprotectant. With an increase in the sucrose concentration and prolongation of the pretreatment time, *P. serbia* could be successfully cryopreserved under air-dried condition even without the use of any cryoprotectant [26].

4.2. Effect of Cryoprotection Condition on Fresh Weight and Cell Survival Rate of the Callus

DMSO, as a basic cryoprotectant in the cryopreservation process, can reduce the freezing point of cells, prevent the formation of crystals in the freezing process of cells,

and reduce the degree of damage to cells caused by ice crystals. However, DMSO has a certain toxic effect on cells. Materials with excessive DMSO concentration are prone to browning and even death. Too low DMSO concentration can decrease frost resistance [25]. In a slow cooling cryopreservation study of somatic embryos of *Citrus sinensis*, the relative cell viability was 31% after cryoprotection with 10% DMSO [27]. Another study reported that the cell viability of *F. mandshurica* embryonic callus after cryoprotection for 60 min in 7.5% DMSO was the highest (68%). In the present study, after the embryonic callus of *F. mandshurica* was cryoprotected in 7.5% DMSO for 90 min, the cell viability detected by TTC after recovery culture of *F. mandshurica* reached 71.6%, and the regeneration rate of the callus was 100% after 60 days of culture, indicating that the most suitable cryoprotection time for *F. mandshurica* embryonic callus is 90 min (Figure 5).

In this study, the temperature of the cryoprotection stage was 25 °C, and no other temperature gradient was set. Related studies have reported that cryoprotection at a low temperature of 0 °C can effectively improve the cell viability of tropical plant species that are relatively sensitive to temperature [28]. Under this condition, selecting a suitable cryoprotectant can shorten the cryoprotection time, reduce the exposure of callus, and be more beneficial to the recovery and growth of sensitive plants [29].

In summary, this study explored the relationship among material screening, preculture conditions, concentration of cryoprotectant, cryoprotection time, and the fresh weight and cell viability of callus to identify an appropriate cryopreservation scheme and optimize the slow cooling method for the cryopreservation of *F. mandshurica* embryonic callus. The results indicated that preculture of embryogenic callus in 0.4 mol·L^{-1} sorbitol solution for 20 h at room temperature, followed by its cryoprotection in 7.5% DMSO solution at 0 °C for 90 min, is the optimal material treatment method. The freezing tube was placed in a −80 °C refrigerator for 2 h and then quickly put into liquid nitrogen for frozen storage. During thawing, the cryopreservation tube was taken out from liquid nitrogen, directly placed in a water bath at 40 °C for 2 min, and used for culturing on the medium WPM + 0.1 mg/L6-BA + 0.15 mg/L2, 4-D. The findings may provide a theoretical basis for solving the problems concerning embryogenic loss of *F. mandshurica* embryonic callus and long-term conservation of germplasm resources.

Author Contributions: L.Y., H.S., I.N.T. and A.M.N. conceived and designed the study. X.Y. and Y.L. collected plant materials and prepared samples for analysis. X.Y., Y.L. and X.L. analyzed the results for experiments. L.Y. and X.Y. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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