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Cryopreservation of winter-dormant mulberry buds using two-step freezing

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Abstract

Genetic resources of mulberry trees are commonly preserved as trophosomes, which are vulnerable to environmental factors, such as natural disasters, diseases, and pests. This study establishes a basic protocol for ultra-low temperature cryopreservation of mulberry trees using a two-step freezing process. The procedure was established using the "Daeshim" variety and then tested on genetic resources from 24 other mulberry varieties. Samples were first dried to a moisture content of 33–43% in a low-temperature forced-air chamber at –5 °C, then slowly frozen from –5 °C to –20 °C, and preserved in liquid nitrogen (–196 °C). To determine the regeneration rate, isolated dormant buds were inoculated into MS basal medium, and grown shoots were grafted onto 1-year-old rootstock via chip budding and then cultured. After freezing in liquid nitrogen, the "Daeshim" variety exhibited a survival and regeneration rate of more than 70% and 50%, respectively. Applying the two-step freezing process to genetic resources from 24 mulberry species yielded average survival and regeneration rates of 85.3% and 75.5%, respectively. *Morus alba* showed survival and regeneration rates of 100%, confirming the efficacy of the two-step freezing method. These results indicate the high feasibility of ultra-low-temperature cryopreservation through two-step freezing of dormant buds from mulberry genetic resources. Additional research is required into the variations in regeneration rates with freezing period in liquid nitrogen.

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Introduction

The mulberry tree is a deciduous broad-leaved tree belonging to the Moraceae genus of the Moraceae family and is cultivated in Asia. Mulberry trees have mainly been used as a food source for silkworm breeding in the sericulture industry (Rahul *et al.,* 2022; Jeong *et al.,* 2022; Kim *et al.,* 2021). To this end, plant resources have been collected, and varieties have been described. Among countries with active sericulture, China has the largest number of genetic resources (2,600 lines), followed by Japan with 1,375 lines, India with 1,109 lines, Korea with 615 lines, and Bulgaria with 140 lines (Ananda *et al*., 2009). However, the genetic resources of mulberry trees can only be preserved in the form of vegetative bodies, which makes management

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difficult because of the high maintenance costs of cultivation and management. Additionally, vegetative bodies are vulnerable to natural disasters and pests.

In the past, long-term cryogenic storage technology using liquid nitrogen was widely used to preserve fertilized eggs and animal cells, whereas plants were preserved through seed storage or germpalsm preservation. However, the development and commercialization of ultra-low-temperature cryopreservation is receiving much attention, from both breeders and growers, as an important means of quickly responding to changing market demands (Halmagyi *et al*., 2004; Benson and Withers, 1998). When organisms are preserved at ultralow temperatures, the metabolic process is halted (Engelmann, 2004; Fatima *et al*., 2009), allowing genetic resources to be preserved for a long period, at a lower management cost, without the loss of genetic characteristics.

As awareness around the importance of ultralow-temperature preservation increases, research is being conducted on how best to apply this method to various plants for which germpalsm genetic resources have been preserved. Chrysanthemum is a typical example, and cryogenic preservation has been achieved through slow-freezing by controlling the cooling rate (Fukai, 1990; Halmagyi *et al*., 2004), pre-cultivation (Hitmi *et al*., 1999), and encapsulation-dehydration by alginic acid coating and dehydration treatments (Sakai *et al*., 1990 and 2000; Halmagyi *et al*., 2004; Martin and Gonzalez, 2005), as well as droplet-freezing using aluminum foil (Halmagyi *et al*., 2004). Long-term ultralow-temperature preservation has been attempted using methods such as vitrification by dehydration in a high-concentration vitrification solution (Ahn and Skai, 1994; Sakai *et al*., 2000; Halmagyi *et al*., 2004; Martin and Gonzalez, 2005). In addition, woody apple and persimmon plants have been successful regenerated through a two-step freezing and cryopreservation process using dormant buds and liquid nitrogen (Yi *et al*., 2013; Matsumoto *et al*., 2001). However, there have been too expensive of ultra-low-temperature cryopreservation of dormant buds in woody plants, and research is lacking on the cryopreservation of mulberry trees.

This study examined the possibility of a two-step freezing method for the ultra-low temperature cryopreservation of dormant buds of 'Daeshim' (*M. alba*), a representative mulberry-producing variety in Korea, was used. 'Daeshim' is a variety developed in 2014 that was characterized by very high productivity and large fruits (Ju *et al*., 2018). This was conducted with the aim of efficiently managing the genetic resources of the mulberry tree and determining requirements for applying ultra-low temperature cryopreservation to the available genetic resources.

Materials and methods

Sample collection

Samples were collected in field at the Ministry of Agriculture and Biology of the Rural Development Administration, Wan-Ju, Korea, early January 2022 for the establishment of drying and freezing conditions, and early January 2023 for analysis of the regeneration of 24 genetic resources. The stems were collected after five days of sub-zero temperatures. Collected scions were wrapped and stored at –5 °C for cold acclimation for two weeks.

Sample processing

Scions were cut into single- or two-node sections, each 100 mm long, with the buds in the central position. They were classified according to their cross-sectional diameters into S (less than 10 mm), M (10–14 mm), and L (>14 mm) groups. The scions were then spread out on a tray, left unsealed, and dried using a low-temperature forced-air dryer (Dasol Scientific, Hwaseong, South Korea) by cooling to −5 °C for 24 h and cooling to −20 °C for 4 days. Fifteen twig samples were prepared from each of the three size groups of dried scions, and the moisture content was measured using the atmospheric-pressure heating drying method. When branches reached the target moisture content, they were packaged in polyolefin tubes and quickly frozen in liquid nitrogen for at least 24 h. Afterwards, sections were removed from the liquid nitrogen and gently thawed in moist peat at 4 °C for 24 h. To analyze the regeneration rate of 24 genetic resources, only branches with on bud of M-size were selected and the same drying process as above was performed to reduced moisture content to 37~42%. Afterwards, it was frozen for 24 h using liquid nitrogen and thawed at 4 °C.

Viability assessment

To determine the viability of thawed dormant buds, they were stained by immersion in 1% tetrazolium blue chloride (Sigma Aldrich, St. Louis, USA). To check for regrowth of the surviving dormant buds, thawed sections were immersed in 1.25% NaOCl for 15 min to disinfect the surface and cultured in hormone-free Murashige and Skoog medium (MP BIOMEDICALS, Solon, OH, USA) for 2 weeks. Germination was completed

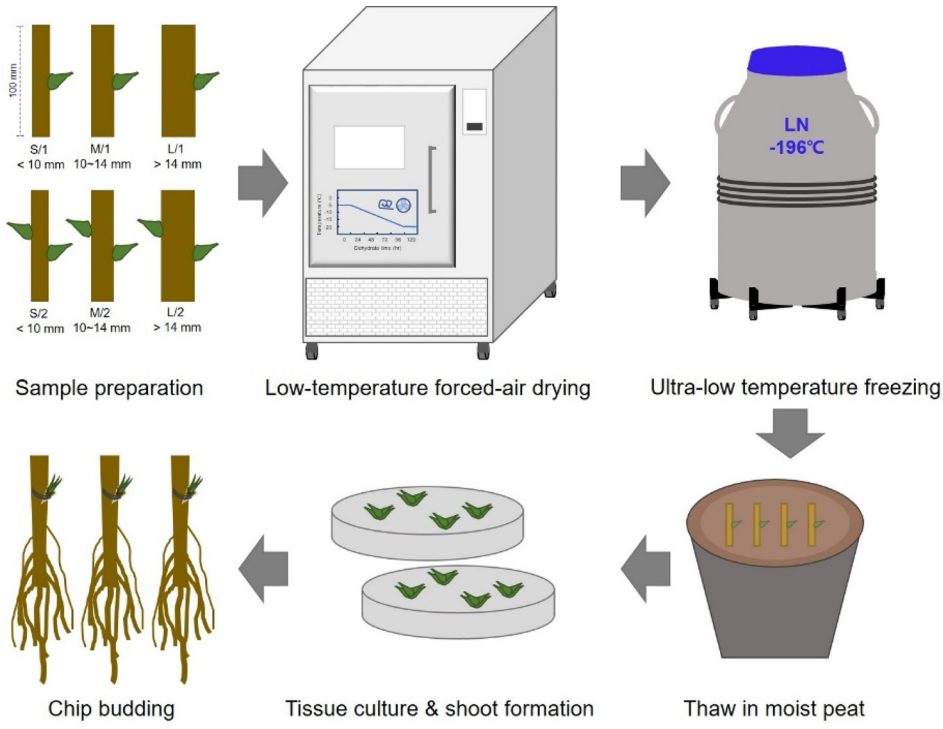

Fig. 1. Schematic diagram of the two-step freezing and cryopreservation process for dormant mulberry buds. After trimming the mulberry branches, they are cooled to −20 °C using a low-temperature forced-air drying machine and dried to the desired moisture content. Branches containing dried dormant buds were packaged in moisture-proof film and then immersed in liquid nitrogen. After 24 h, branches were removed from liquid nitrogen and buried in moist peat and thawed at 4 °C. Dormant buds were extracted from thawed tree branches and cultured on solid medium to obtain new shoots, which were grown by performing chip budding on 1-year-old rootstock.

by transferring the seeds to Murashige and Skoog medium supplemented with 1.5 mg/L of 6-Benzylaminopurine (MP BIOMEDICALS, Solon, OH). Grafting was performed using a chip budding technique with a 1-year-old mulberry rootstock. The sprouted rootstocks were kept in a greenhouse, and shoot growth was examined over 2 months. Viability was assessed based on bud formation from the grafted shoots.

Statistical analysis

All experimental results are expressed as mean value ± standard error (SE), and one-way analysis of variance (ANOVA) was conducted using IBM SPSS Statistics 23 (IBM, USA). The significance of each average value ($p<0.05$) was determined using Duncan's multiple-range test.

Table 1. Differences in water content after drying, and survival rate before and after freezing in liquid nitrogen, according to the thickness of mulberry branches.

Size*/ Bud No.	Water content (%)	LNC***	LN
		survival (%)	survival (%)
Fresh / 1	48.3±5.2^{a**}	95±6.^{a}	0±0^{c}
S / 1	32.6±3.3^{bc}	90±4.5^{a}	38.6±6.2^{b}
M / 1	34.1±1.2^{b}	95±4.2^{a}	82.9±5.2^{a}
L / 1	32.5±6.4^{bc}	50±8.2^{b}	0±0^{c}

*Section diameter categorized as S < 10 mm; M 10–14 mm; L >14mm.
**The values represent Mean±SD for triplicate experiments (n=15). Means with same letters are not significantly different at $p<0.05$ according to Duncan's multiple range test.
***LNC and LN refer to be before and after liquid nitrogen treatment, respectively.

Results and Discussion

Basic protocol settings for two-step freezing

The two-step freezing protocol designed for the ultra-low-temperature cryopreservation of dormant mulberry buds is shown in Fig. 1. Although the samples had a moisture content of 48% prior to drying, branches in the S, M, and thicknesses groups had moisture contents of 32.6, 34.1, and 32.5%, respectively (Table 1). For the

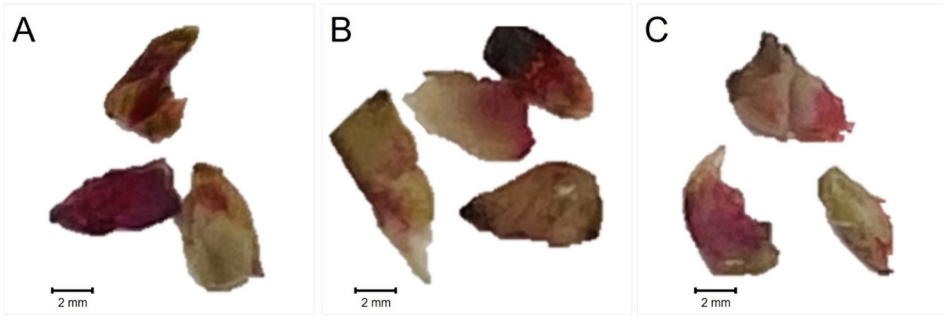

Fig. 2. Testing for survival of dormant buds using tetrazolium staining. (A) Fresh dormant buds, (B) dormant buds before cooling in liquid nitrogen, and (C) dormant buds after freezing and thawing using liquid nitrogen.

S and M branches, the drying efficiency decreased as the thickness increased, confirming that the moisture content of M branches was high. However, the resulting moisture content of the L branches was similar to that of S branches, despite their thickness. This is attributed to the low efficiency of the low-temperature forced-air drying process; however, it was judged not completely dried during the moisture content measurement process.

To determine their survival, dormant buds from the dried branches were separated and stained with tetrazolium (Fig. 2A and B). The survival rate of dormant buds in fresh branches was 95%, and the survival rate of dormant buds from M-sized branches was 95%, similar to that of live branches; the L-sized branches had a survival rate of approximately 50% (Table 1). Most of the dormant buds from the M-sized branches were stained red (Fig. 2C), with a survival rate of 82.9%. However, all dormant buds present in undried and L-size branches were not stained (Table 1). Based on these results, tree branches were judged as suitable for cryopreservation if they had a thickness of 10–14 mm and a moisture content of 32% or more.

According to Forsline *et al*. (1998), some cold-tolerant species such as *Malus baccata* and *Prunus virginiana* can be frozen and preserved without drying. However, according to the US National Center for Genetic Resources Preservation (NCGRP), the most common cryopreservation method is pollination of scions harvested in winter. Scions were dried until the moisture content reached 30%, and then cooled in two stages and stored in liquid nitrogen (Towill and Bonnart, 2005). According to Forsline *et al*. (1998), the process of daily drying and measuring moisture content for cryopreservation consumes a lot of time and effort, the moisture that has been released during the winter and dried is suitable for cryopreservation. In addition, according to Sakai (1960), it was reported that freezing speed also affects the survival rate of dormant buds in mulberry trees. Therefore, if samples for cryopreservation are prepared by keeping the harvest time of the buds, thickness of the branches, and drying time constant, as in this study, this can be advantageous for mass processing.

Effect of number of dormant buds and moisture content

To analyze the effects of the number of dormant buds and moisture content on regeneration, the moisture content and survival rate were determined for branches of different thicknesses with one or two dormant buds, after varying drying times (Table 2). Before freezing in liquid nitrogen, dormant buds were largely stained with tetrazolium regardless of the branch thickness or moisture content, and showed similar survival rates. However, after freezing in liquid nitrogen, M-sized branches dried to approximately 38% moisture content showed the highest survival rate of 80.6%. The survival rate of these dormant buds was similar to that of M-size branches with a moisture content of 34.1% and one dormant bud (Table 1). The number of dormant buds did not have a significant effect on the survival rate during freezing. The appropriate moisture content for drying was determined to be 34–40%.

To analyze the regeneration rate of thawed dormant buds, cut dormant buds were cultured on a solid medium (Fig. 3), and shoots were grafted onto the rootstock and cultured (Fig. 4). Shoots cultured before freezing with liquid nitrogen had a regeneration rate of 90%, regardless of branch thickness or moisture content (Table 2). However, after freezing and thawing with liquid nitrogen, the regeneration rate rapidly decreased to less than 50%, and reached a maximum of only 55.2% for the M-size group with a moisture content of 38.5%,

Table 2. Effect of water content and number of dried dormant mulberry buds on survival, before and after liquid nitrogen treatment.

Size* / Bud No.	Water content (%)	LNC****		LN	
		survival (%)	regeneration (%)	survival (%)	regeneration (%)
Fresh / 2	48.3±5.2^{a**}	89.9±6.8^{c}	88.8±5.9^{b}	-***	-
S / 2	37.7±1.3^{c}	100±0.0^{a}	74.8±6.2^{c}	72.15±4.4^{c}	32.9±3.3^{cd}
S / 2	41.4±2.3^{b}	100±0.0^{a}	96.5±4.5^{a}	67.5±3.3^{d}	36.7±2.9^{c}
M / 2	38.5±2.3^{bc}	97.5±1.8^{ab}	93.5±4.8^{a}	80.6±3.2^{a}	55.2±3.1^{a}
M / 2	41.5±2.1^{b}	100±0.0^{a}	95.3±5.1^{a}	77.8±2.8^{ab}	49.1±2.7^{b}

*Section diameter categorized as S < 10 mm; M 10–14 mm
**The values represent Mean±SD for triplicate experiments (n=15). Means with same letters are not significantly different at $p<0.05$ according to Duncan's multiple range test.
***Indicates no value because no analysis was performed.
****LNC and LN refer to be before and after liquid nitrogen treatment, respectively.

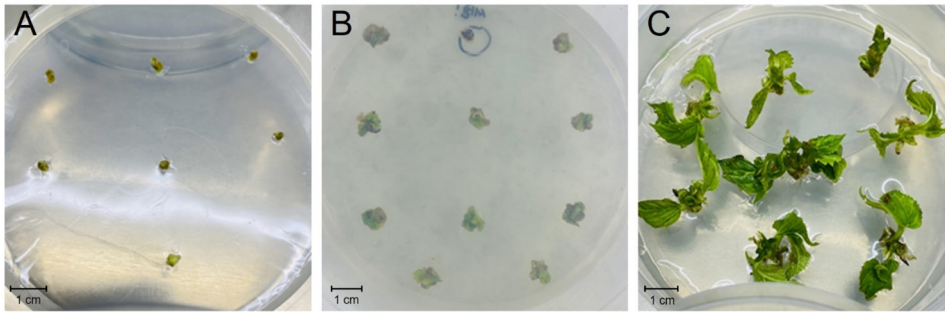

Fig. 3. Cultivation and regeneration of dormant buds for shoot production. Disinfected dormant buds were (A) inoculated into hormone-free MS medium, (B) cultured at 25 °C for 2 weeks, then transferred to (C) MS medium containing 1.5 mg/L of 6-Benzylaminopurine and incubated at 25 °C for 2 weeks, resulting in shoots.

Fig. 4. Chip budding and culture for regeneration of frozen dormant buds. (A) From the shoots cultured in MS medium, healthy ones were selected and chip budding was performed on rootstocks with a diameter of approximately 10 mm. (B) The grafted rootstock was transplanted into moist peat and grown in a greenhouse for 30 days, and (C) after 100 days, the grafted plants had completely regenerated and grown substantially.

(Table 2). Yi *et al*. (2013) found that two-step freezing of apple dormant buds, followed by grafting, returned a regeneration rate of 40–85%, depending on the conditions, and that moisture content plays an important role in ensuring a high survival rate after cryopreservation. Towill *et al*. (2004) also reported a high regeneration rate in properly dried scions, suggesting that drying before freezing is important. Therefore, we determined that the moisture content required for the proper cryopreservation of mulberry trees is 34–40%, which necessitates appropriate drying of fresh branches with their moisture content of 48%. Although substantially lower survival rates were achieved with dormant mulberry buds preserved in liquid nitrogen (without previous drying), this survival rate of approximately 55.2% was deemed to be acceptable for cryopreservation.

Analysis of regeneration rate according to mulberry genetic resources

To identify any difference in survival rate between different

Table 3. Analysis of regeneration rates after two-step freezing of dormant mulberry buds from genetic resources

Genetic resources	Scientific name	Water content (%)	LNC		LN	
			Survival (%)	Regeneration (%)	Survival (%)	Regeneration (%)
'Daeshim'	*M. alba*	41.5*	-**	-	73.3	65.0
Mujeonsibmunja	*M. alba*	38.3	100	100	94.4	65
Cheong-unppong		41.8	-	-	100	100
Suwonppong		40.6	90.9	90.9	100	100
Yongcheonppong		39.2	-	-	95	90
Cheongmogsipyeong	*M. bombycis*	40.1	-	-	95.8	87.8
Jeolgogjosaeng		36.5	100	100	90	85
Bbulgugsang		34.6	66.7	66.7	83.3	55.6
Sasang	*M. lhou*	37.3	-	-	96.4	88.7
Ilpummog		40.5	100	100	88.9	72.2
Milseongppong		39.9	-	-	70	60
Daelyugppong		41.1	-	-	100	80
Baegpihyeongsang		35.9	-	-	66.7	54.2
Jeong-yasang		40	91.7	91.7	83.8	72.2
Bupyeong	*M. sp.*	40.3	-	-	86.4	76.4
Jangloe		37.9	-	-	95.8	90.8
Sangjeongab		38.5	75	75	57.1	42.9
Daeyeobgomog		39.1	75	75	55.6	55.6
JangjamB		40.6	83.3	83.3	77.4	69
Jeoggabchan		40.6	83.3	83.3	63.3	58.3
Sujungsang		39.4	-	-	100	95.8
Baeg-un3ho		39.5	-	-	91.7	83.3
Yangmyeonjosaeng		39.2	-	-	95	80
Osaengsibmunja		37.3	-	-	100	91.7
Bugwigeum		40	100	100	60.8	57.1
Mean		39.1	87.8	87.8	85.3	75.5

*The values represent mean values obtained from triplicate experiments (n=15).
** indicates no value because no analysis was performed.

mulberry varieties, samples of mulberry genetic resources placed under the conditions established in 2022 were collected from 24 genetic resources in 2023, and two-step freezing was performed to determine survival and regeneration rates. The results are shown in Table 3. All mulberry branches had an M-sized thickness, and the moisture content after drying was adjusted to 34–40%. The after freezing in liquid nitrogen and subsequent thawing, 'Daeshim' showed a regeneration rate of approximately 65%, whereas in the 'Cheong-unppong' and 'Suwonppong' varieties exhibited complete regeneration rates of 100%. 'Sangjeongab' was confirmed to have the lowest regeneration rate among the genetic resources analyzed, of only 42.9%. For the 25 samples analyzed in 2023, the average survival rate after liquid-nitrogen freezing and thawing was 84.8%, and the regeneration rate of surviving dormant buds was 75%, which was approximately 20% higher than that in the large trial conducted

in 2022 (Tables 2 and 3). This substantial increase in regeneration rate compared to the increase in survival rate after thawing is due to the improvement in grafting technology using cultured shoots by the researcher who conducted the analysis. In addition, the survival and regeneration rates of *M. alba* were characteristically high, and there were other significant differences between the species. According to Sung *et al.* (2002), *M. alba* species have high cold tolerance and inhabit various regions around the world. Therefore, species with higher cold resistance are more likely to survive and regenerate successfully after cryopreservation.

Based on these results, we conclude that the two-step freezing technology using dormant buds is applicable for performing ultra-low-temperature cryopreservation of mulberry genetic resources. The optimal conditions obtained were tree branches with diameters of 10–14 mm and a 34–40% moisture content, slowly frozen from −5 °C to −20 °C, and after freezing using liquid nitrogen, the regeneration rate ranged from a minimum of 54.2% to a maximum of 100%. has been confirmed. However, skilled researchers are required to regenerate cryopreserved genetic resources successfully, and since large numbers of dormant buds must be preserved to offset the low regeneration rate, sufficiently large storage facilities must be established (Lambardi, 2012). In addition, because thawing was performed 24 h after freezing with liquid nitrogen, additional analysis of survival and regeneration rates according to the storage period is necessary to optimize long-term storage of dormant mulberry buds at ultra-low-temperatures.

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