In Vitro Storage of Hop (*Humulus* L.) Germplasm

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Keywords: cryopreservation, cold acclimation, cold storage, *Humulus lupulus*, liquid nitrogen, tissue culture

Abstract

In this study we characterized the response of 70 diverse *Humulus* L. genotypes from the USDA-ARS National Clonal Germplasm Repository, Corvallis, Oregon, to in vitro storage under low light at 4° C and 28 to cryopreservation in liquid nitrogen by slow cooling. Plantlets were cold acclimated, stored in plastic tissue-culture bags, and evaluated at 4 mo intervals. The average storage time without transfer for the 70 genotypes evaluated was 14.1 ± 3.5 mo with a range of 6 to 26 mo. Eight accessions stored for 2 cycles averaged 14.3 ± 3.2 months storage. Plantlets for cryopreservation were cold acclimated for 2 wks, pretreated for 48 hrs, cooled to -40° C at 1° C per min, and plunged into liquid nitrogen. Mean recovery of cryopreserved shoot tips of accessions was 54% ± 13 with 2-wk cold acclimation. A few genotypes required longer cold acclimation for good recovery following liquid nitrogen exposure.

INTRODUCTION

Hops germplasm collections are commonly held in field collections as perennial rhizomes. Diseases, insects, and environmental stresses put these plants at risk and virus diseases can accumulate in a field collection and might be transferred to additional sites by vegetative propagation. The USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, Oregon, stores nearly 700 accessions of *Humulus* germplasm collected from many sources. The accessions are held as a field collection, 62 representative (core) accessions were heat treated to eliminate viruses and are stored in pots under screen, and as in-vitro cultures. In-vitro storage can provide safe medium-term storage for germplasm distribution. Cryopreservation is now considered the only viable option for long-term (base) storage of clonally propagated germplasm (Ashmore, 1997). This study characterizes the response of diverse genotypes of *Humulus* germplasm to in-vitro cold storage and to cryopreservation in liquid nitrogen following established techniques (Chang and Reed, 1999; 2000; 2001; Reed, 1999; 2001; Reed et al., 1998). Our objective was to store cryopreserved accessions as a base collection and to determine the response of clonally propagated *Humulus* germplasm to standard medium- and long-term storage techniques.

MATERIALS AND METHODS

Plant Materials

In vitro cultures were initiated from 0.3 to 0.5 mm meristems of heat-treated shoots from clonally propagated hops plants (Adams, 1975). *Humulus lupulus* L., *H. lupulus var. lupuloides* E. Small, *H. lupulus var. neomexicanus* A. Nelson & Cockerell, *H. lupulus var. pubescens* E. Small, and *H. japonicus* Siebold & Zucc. were included in the study (Table 1). At initiation, meristems were cultured individually in 24-well culture plates on 2 ml NCGR-HUM medium composed of Murashige and Skoog (1962) salts and vitamins with 2% glucose, 4.4 μM N^6 benzyladenine, at pH 5.0 and gelled with 0.3% agar and 0.125% Gelrite. New shoots were transferred to 10 ml fresh medium in 16 x 100 mm tubes after 3 to 4 wks or to 40 ml of medium in Magenta GA-7 vessels for additional growth and multiplication. Cultures were grown at 25° C under a 16-h photoperiod (40

µmol·m^{-2}·s^{-1}).

Cold Acclimation (CA)

Mother plants on NCGR-HUM medium were CA for 1 wk for cold storage and 2 wk for cryopreservation in a growth chamber with temperature/photoperiod settings of -1° C 16-h dark/ 22° C 8-h light (10 µmol·m^{-2}·s^{-1}) as the standard treatment (Reed, 1988).

Cold-Storage of In Vitro Cultures

Storage followed the technique developed for other genera but with NCGR-HUM medium (Reed, 1999, 2002; Reed et al., 1998). Plantlets (2 to 3 cm height) were transferred to two 5-chambered semi-permeable tissue-culture bags (Star-pak, Garner Enterprises, Willis, Tex) with 10 ml medium per chamber 3 wk after the last regular subculture. Storage medium was NCGR-HUM medium without growth regulators, and was gelled with 0.35% agar and 0.145% gelrite. Ten plantlets of each accession were stored, each in an individual section (15 x 150 mm) of a five-section bag. Sealed cultures were grown for 1 wk in the growth room, and then for 1 wk under the cold acclimation (CA) conditions described below. Storage was at 4° C with a 12-h photoperiod and very low light (3 µmol·m^{-2}·s^{-1}). Data was taken at 4 month intervals and each bag of plantlets was rated on a 0 to 5 scale. Ratings were: 5, dark green leaves and stems, no etiolation, base green; 4, green leaves and stems, little etiolation; 3, shoot tips and upper leaves green, etiolation present, base green; 2, shoot tip green, leaves and stems mostly brown, base may be brown; 1, plantlet mostly brown, only extreme shoot tip green, much of base dark brown; 0, all of plantlet brown, no visible green on shoot tip. Plantlets were removed for repropagation when ratings reached ≤ 2 and the length of storage was noted (Reed, 1999, 2002; Reed et al., 1998). One storage period was studied for 70 accessions and the 10 plantlets of each accession were treated as a group.

Cryopreservation

Shoot tips (0.8 to 1.0 mm) of 1-wk or 2-wk CA plantlets of each genotype were dissected and precultured on NCGR-HUM medium with 0.35% agar and 0.18% Gelrite and 5% dimethyl sulfoxide (DMSO) for 48 hr under the same temperature conditions as the parent shoots. Samples were subjected to slow cooling (Reed, 1990). Ten shoot tips were transferred to 0.25 ml liquid MS medium in 1.2 ml plastic cryovials on ice and the cryoprotectant PGD (Finkle and Ulrich, 1979), a mixture of 10% (w/w) each of polyethylene glycol (MW 8000), glucose, and DMSO in liquid MS medium, was added drop wise up to 1.2 ml over 30 min (Reed, 1990). After 30 min equilibration on ice, the shoot tips were frozen to –40° C at 0.1° C/min in a programmable freezer (Cryomed, Forma Scientific, Mt. Clemens, Mich.) with nucleation at –9° C and immersed in liquid nitrogen. Vials were thawed in 45° C water for 1 min, then in 25° C water for 2 min. The cryoprotectant was removed and replaced with liquid MS medium. Shoot tips were plated in 24-cell plates with 2 ml NCGR-HUM medium per cell (Costar, Cambridge, Mass.) for recovery. Regrowth data were taken 6 wks after thawing. Each experiment included 20 shoot tips in one vial for each treatment and five shoot tips for unfrozen controls, with at least three replications of the experiment (n = 60). For storage, 150 shoot tips of each accession were processed. Two vials of 25 shoot tips served as cryopreserved controls (one thawed at NCGR and one thawed at the National Center for Germplasm Resources Preservation) (n=50) and 10 vials of 10 shoot tips (n=100) were stored as a base collection.

RESULTS AND DISCUSSION

Cold Storage of In Vitro Cultures

Plantlets of 70 accessions remained in one cycle of cold storage for an average of 14.1 ± 3.5 months (Reed et al., 2003). The range for individual accessions was 6 to 26 months. There were no significant differences in length of storage for cultivars (14.6 ±

3.4) vs. wild accessions (12.6 ± 3.2). Ratings decreased by about one point for each inventory period for the majority of plants. Long-storing plants remained at the "3" rating level (shoot tips and upper leaves green, etiolation present, base green) for longer periods than the average plant. This technique was originally optimized for *Fragaria* (18 months ± 6), and was then applied to *Rubus* (27 months ± 14), and *Pyrus* (32 months ± 12) (Reed, 1999, 2002; Reed et al., 1998). Variation also may occur between storage cycles due to many factors. Eight accessions studied for two storage cycles produced a mean storage time of 14.4 ± 2.6 months for the first cycle, 14.3 ± 5.9 months for the second, with an average of 14.3 ± 3.2 months for the 2 cycles (Fig. 1). Additional study will be needed to determine the cause of this variation, however the growth condition of the plantlets, the personnel involved and the medium used can all be factors in the length of cold storage for an accession (Ashmore, 1997). *Humulus* has a very different growth form from other plants stored in this fashion and is in the Cannabinaceae, a family quite distinct from the Rosaceous genera for which the method was developed. Despite these differences the *Humulus* accessions remained in good condition for an average of more than one year. This result was confirmed by a second storage period. It is likely that additional improvements can be made to increase the length of storage for individual *Humulus* accessions, but for the majority the technique is directly applicable. When optimizing a medium-term in-vitro storage system the age, size and physiology of the plants must be considered as well as temperature of storage, cold acclimation, roots, single or multiple shoots, light intensity and quality and storage medium (Ashmore, 1997; Moriguchi and Yamaki, 1989; Reed, 1999; 2002; Reed and Chang, 1997; Reed et al., 1998). Individual factors require long periods of study to fully optimize storage for a genus; however, the standard technique can be used quickly and with generally good results almost immediately following the decision to store a new type of plant. With proper monitoring of the stored collection at regular intervals, even the poorest performing genotypes can be safely held.

Cryopreservation

The slow-cooling procedure developed for *Pyrus* (Chang and Reed, 2000; 2001; Reed, 1990; Reed et al., 1998) was successful for all accessions tested. We consider 40% the minimum acceptable recovery for storing accessions and most of the initial accessions tested exceeded that minimum. The 40% minimum was chosen based on our experience with the variation possible between tests. Longer cold acclimation (2 to 12 wks) is necessary for some genotypes of *Pyrus* and *Rubus* (Chang and Reed, 1999; 2000; 2001). All *Humulus* cultivars and wild accessions tested with 2-wks CA recovered at acceptable rates for storage (regrowth $\geq$ 40%) (Fig. 2A, 2B). A recent study of cryopreservation of *Humulus* shoot tips found that slow cooling following 1 to 6 wks of a 12 °C/ 6 °C CA regime and DMSO-sucrose cryoprotectants produced no surviving shoot tips; a vitrification technique was also unsuccessful while the encapsulation-dehydration technique was very successful for the accessions tested (Revilla and Martinez; 2002). Our preliminary studies with encapsulation-dehydration were also successful and varied with genotype in the same recovery range as the slowly-cooled samples (Fig. 2C). Our study shows that success of various cryopreservation techniques requires attention to critical points of the protocols. In the case of slow-cooling techniques, the type and length of CA and the cryoprotectant used are extremely important as well as the cooling rate. Deeper cold hardiness and increased regrowth in pear following cryopreservation are produced by alternating-temperature CA treatments that expose the plantlets to freezing temperatures (-1° C) and shorter warm periods (22° C) (Chang and Reed, 2000). Extended alternating-temperature CA periods of 2 or more weeks may also be needed for reaching optimum cold hardiness in some *Humulus* accessions. The efficacy of PGD as a cryoprotectant for use in slow-cooling protocols is clearly demonstrated for cells and shoot tips of numerous genera (Chang and Reed, 1999; 2000; 2001; Finkle and Ulrich 1979; Reed; 1988; 1990; 1999; Reed and Chang, 1997).

Storage of large germplasm collections requires protocols that work well for the

particular lab involved and that are successful for many types of plants. As germplasm curators prioritize the type and amount of germplasm to store, they also need to choose a technique that fits their facility. Personnel, equipment, expertise, plant type and available facilities influence which technique is most appropriate for a particular facility (Reed, 2001). Cold storage of in-vitro collections is very labor efficient and keeps the germplasm available for distribution. Cryopreservation is most suitable for long-term storage. When personnel are the rate limiting factor, slow cooling is a time, labor, and financially efficient method for cryopreserved storage of large numbers of accessions. Choosing a well-tested technique and applying it to a new genus can save development time and speed up storage of important plant collections. Base (long-term) germplasm preservation is now in progress for the entire *Humulus* core collection (90+ accessions).

ACKNOWLEDGEMENTS

Funding for this study was provided by the Hop Research Council and USDA-ARS CRIS 5358-21000-033-00D.

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Tables

Table 1. Species and botanical varieties of *Humulus* used in cold storage and cryopreservation studies including plant introduction (PI) number, local identifying number (Local) for NCGR Corvallis and cultivar or accession name (plant name). Further plant information is available at US National Plant Germplasm Web Site: www.ars-grin.gov.

PI number	Local	Plant Name	PI number	Local	Plant Name
Humulus lupulus L.			*Humulus lupulus* L.		
558718	158.004	Backa	558682	197.002	Tettnanger
558653	114.002	Blue Northern Brewer	518760	38.003	USDA 19058M (EG x XS)
302781	130.002	Brewer's Gold	558561	13.004	USDA 19085M (LhS x GCI-Fu S)
558668	132.004	Bullion 10A	558598	53.003	USDA 19173M (SSP x LCS)
558728	175.002	Calicross	558567	19.002	USDA 21072M
558681	196.002	Cascade	558864	716.002	USDA 21120
617364	892.002	Cicero	559069	372.002	USDA 21121 (19005 x 19046M)
558601	56.002	Comet S (Comet x OP)	559063	365.002	USDA 21125 (19005 x 19046M)
558868	720.002	Crystal	559070	373.002	USDA 21127 (19005 x 19046M)
558947	134.004	Eroica	558602	58.002	Wild Yugoslavian 17/17
558664	812.003	Fuggle H	558607	63.002	Wild Yugoslavian 3/3
558692	241.003	Fuggle Tetraploid	558665	128.003	Willamette
617402	930.003	Furano Ace	617392	920.004	Wuerttenberger
558689	211.003	Golden Star	558731	182.002	Wye Challenger
617291	819.006	Hallertauer Gold	558708	144.003	Wye Saxon
617287	815.002	Hallertauer Magnum	558632	88.004	Wye Target
558736	203.002	Hallertauer Mittelfruher	250809	204.003	Yugoslavia Golding
617292	820.005	Hallertauer Tradition	558564	16.002	Zattler seedling
558761	613.002	Hersbrucker-8	558594	49.002	Zattler seedling 2L118 x OP
558793	645.002	Hersbrucker-alpha	*Humulus japonicus* Siebold & Zucc.		
558946	126.003	Huller [Hueller Bitterer]	559274	579.003	Tug Fork # 11 (Kentucky)
559045	246.003	Kirin II	*Humulus lupulus* var. *lupuloides E.* Small		
617387	915.004	Kitamidori	559265	570.002	BC-11 Montana (Wild American)
558685	205.002	Landhopfen	559220	504.002	Millville, Iowa (Wild American)
558559	11.003	Late Cluster seedling	559205	509.001	Millville, Iowa (Wild American)
558621	77.004	Late Cluster seedling	559232	542.002	Montana (Wild American)
558869	721.002	Liberty	558930	782.003	North Dakota 2 (Wild American)

558795	647.002	Lubelska	559200	496.002	Rulo, Nebraska (Wild American)
535808	597.002	Mt. Hood	558589	44.002	Wisconsin selection (Wild American)
558710	146.002	Northern Brewer	*Humulus lupulus var. neomexicanus* A. Nelson & Cockerell		
558667	530.002	Perle	558604	60.003	Arizona 1-2 (Wild American)
558683	198.002	Precoce de Bourgogne	558591	46.002	Colorado 1-1 (Wild American)
558712	148.002	Pride of Kent	558556	8.003	Colorado 2-1 (Wild American)
558783	635.002	Saazer 36	558646	106.001	Colorado 3-1 (Wild American)
255973	201.002	Savinja Golding	558651	112.003	Colorado 7-2 (Wild American)
264597	191.002	Shinsuwase	558616	72.001	Utah 526-5 (Wild American)
617288	816.002	Spalter Select	558625	81.003	Wyoming 2-3 (Wild American)
558666	129.001	Styrian Golding	*Humulus lupulus var. pubescens* E. Small		
617286	814.002	Sunbeam	559213	489.002	Brownville, Nebraska (Wild American)
558774	626.002	SuperAlpha	559198	492.001	Brownville, Nebraska (Wild American)
558678	193.004	Swiss Tettnanger	558635	91.002	Wyoming 3-1 (Wild American)
558677	192.003	Tardif de Bourgogne	558904	755.002	Missouri 3 (Wild American)

Figures

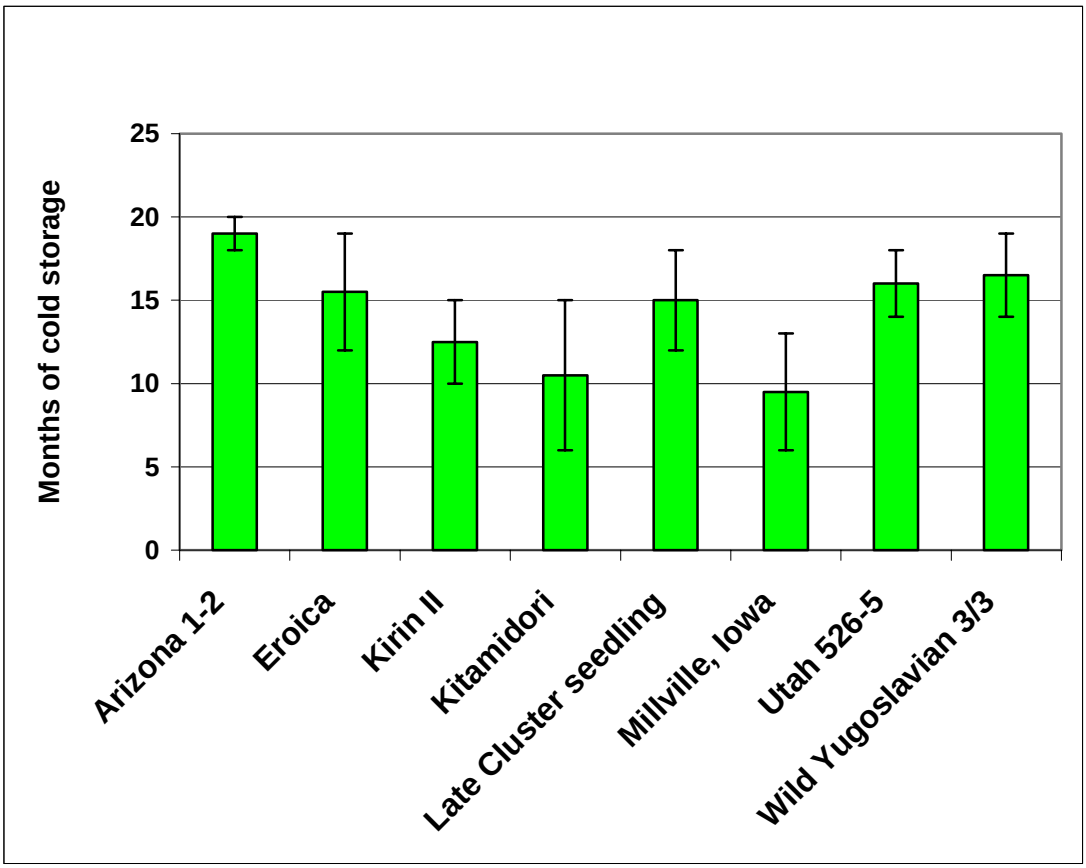
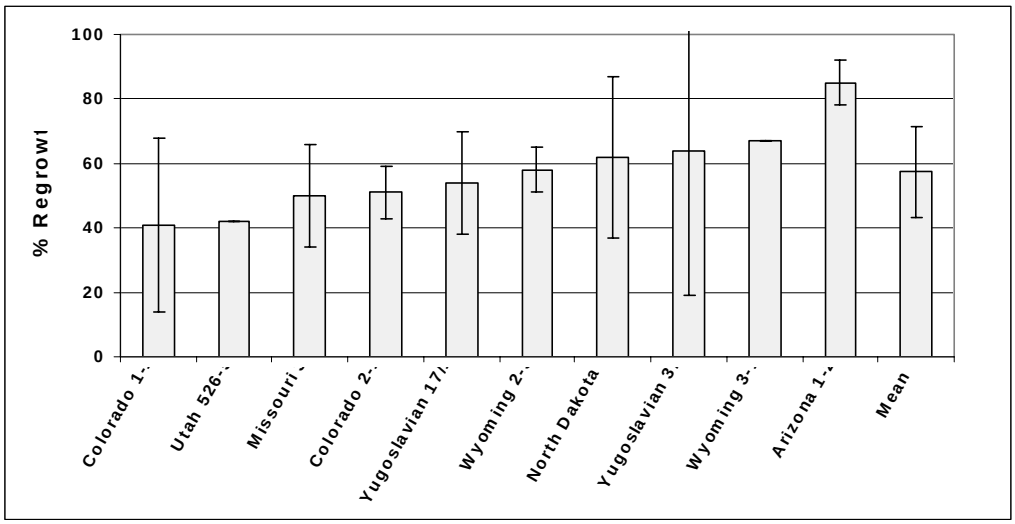
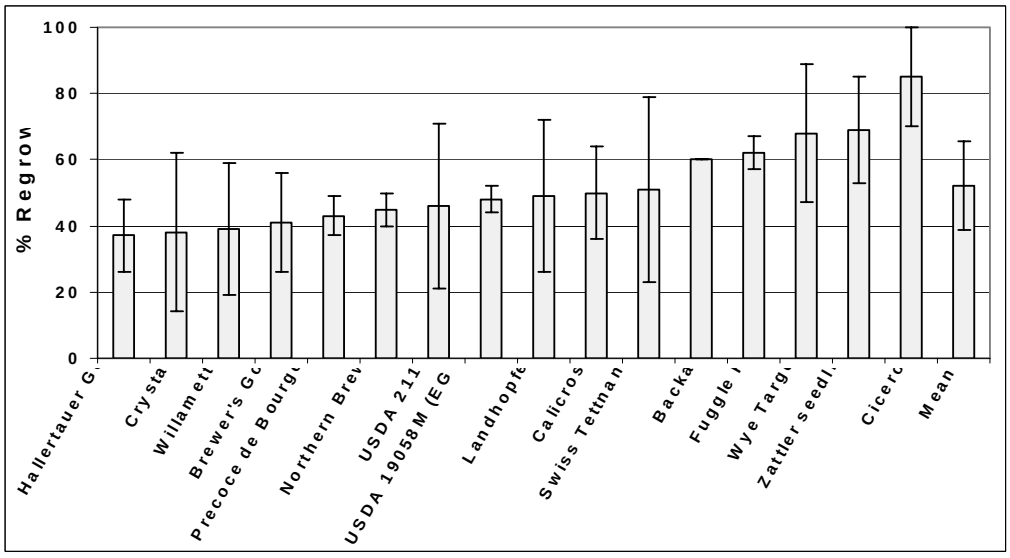


Fig. 1. Mean storage duration of 8 *Humulus* accessions monitored for two cold-storage cycles. Each cycle ended when the plant-condition rating declined from 5 to 2.



A



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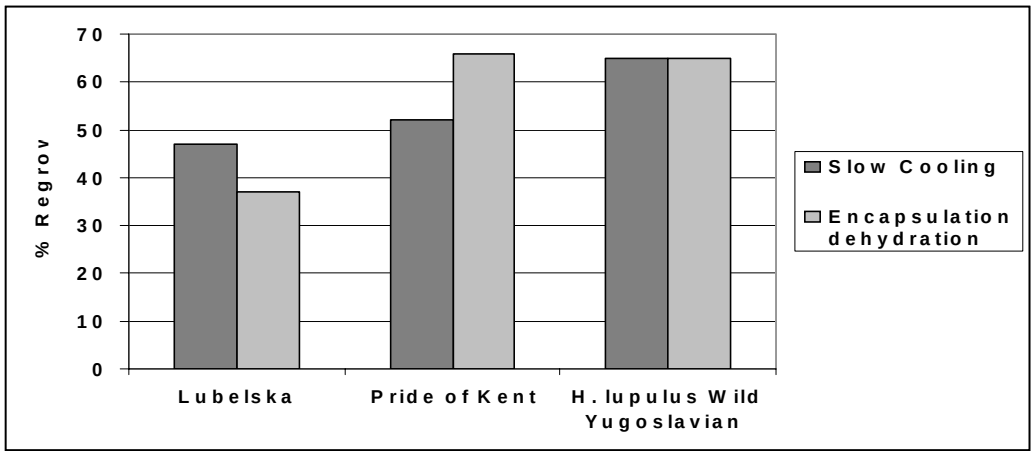

C

Fig. 2. Mean regrowth and standard deviation 6 wks after rewarming for 10 cryopreserved wild *Humulus* accessions (A) and 17 *H. lupulus* cultivars (B) tested 2 to 3 times (n=40 to 60) using 2-wks cold acclimation and cryopreserved by the standard slow cooling technique (Reed et al., 2003). Regrowth of three *H. lupulus* accessions (C) following either slow-cooling or encapsulation-dehydration cryopreservation techniques.