

COLD ACCLIMATION IMPROVES RECOVERY OF CRYOPRESERVED GRASS (*ZOYSIA* AND *LOLIUM* SP.)

Yongjian Chang¹, Reed E. Barker² and Barbara M. Reed^{3*}

¹Department of Horticulture, Oregon State University, Corvallis, OR 97331.

²USDA/ARS National Forage Seed Production Research Center, 3450 SW Campus Way, Corvallis, OR 97331.

³USDA/ARS National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333.

*Corresponding author. Email: reedbm@bcc.orst.edu

Abstract

Cold acclimation of *Lolium* L. and *Zoysia* Willd. grass cultivars significantly increased regrowth of cryopreserved meristems. One wk of cold acclimation improved recovery following cryopreservation but extended cold acclimation (4-8 wk) resulted in the best regrowth. Cold acclimation also significantly increased the dehydration tolerance of both *Zoysia* and *Lolium* meristems. *Lolium* apices cold acclimated for 4 wk produced 60-100% regrowth following cryopreservation by slow freezing or encapsulation-dehydration. Cold-acclimated *Zoysia* had greater than 60% regrowth following encapsulation-dehydration when beads were dehydrated to less than 22% water content. Non-acclimated meristems of both genera had little or no regrowth. Thawed meristems grew quickly without callus formation and the plantlets produced were transplanted to pots in the greenhouse after 4 to 6 wk. Samples of each cultivar were stored in liquid nitrogen as part of the U.S. National Plant Germplasm System.

Key Words: cold acclimation, dehydration tolerance, ryegrass, genetic resources, liquid nitrogen, meristem, zoysiagrass

Introduction

Lolium perenne L., perennial ryegrass, is the most important temperate forage turf grass and is the main component of large areas of permanent pasture in Europe, Japan, southeastern Australia, New Zealand, and portions of the USA. *Zoysia*, zoysiagrass, is a key forage and turf grass in subtropical and dry areas and is very tolerant to high temperature and water stress. Many important cultivars and clones used in genetic mapping of these two genera require vegetative propagation because of their inability to produce sufficient and viable seed, or because they are outcrossing or heterozygous. Preservation of this clonal germplasm is very important for both research and industry, however preservation of these cultivars in the greenhouse or field can be problematic due to possible contamination from runners or seeds. Many original stocks of early cultivars of grass species are no longer available due to difficulties in preserving these clonal genotypes (8).

Cryopreservation is an excellent method for long-term storage of clonal plant germplasm and is successful for many vegetatively propagated species (34). There are three main cryopreservation strategies: slow freezing (or stepwise cooling) (SF), alginate encapsulation-dehydration (E-D), and vitrification (VIT) (7, 22, 27). Slow freezing is relatively easy but requires freezing equipment, which may limit its application. Encapsulation-dehydration uses sucrose as the only cryoprotectant and no special facilities are needed. Vitrification is simple and quick but highly concentrated cryoprotectants are toxic to some plants (25). These cryopreservation techniques are now available to preserve clonally propagated crops, but not all work equally well for specific genera or genotypes. For example, three *Ribes* species and many pears showed distinct genotype responses to cryopreservation techniques (24, 25). Individual protocols for cryopreservation are specifically developed for many crops such as, pear, apple, yam, potato, peanut, and berries (20).

Grass embryogenic cells and cell suspension cultures have been successfully cryopreserved by slow freezing (11, 31). These cell types are not suitable for germplasm preservation due to possible problems with somaclonal variation (13). Meristems are genetically stable and therefore the best materials for backup of important clonal collections. There is only one report available on grass meristem cryopreservation. *Zoysia* meristems cryopreserved by E-D produced shoots initially, however most meristems showed poor regrowth and did not survive reculture (16).

This study was designed to determine the effect of cold acclimation and dehydration pretreatments on recovery of *Lolium* and *Zoysia* cultivars following cryopreservation. We also tested three common cryopreservation techniques to determine their suitability for *Lolium* and *Zoysia* germplasm storage.

Materials and Methods

Plant materials

A single clone of *Lolium perenne* L. cvs. Elka (Elka), SR 4400 (4400), Linn (Linn), Manhattan (Mann), and SR 4500 (SR) and *Zoysia matrella* L. cvs. Cavalier, DH 96 – 12, Diamond, and *Z. japonicas* Steud cvs. Palisades and El Toro were collected from potted greenhouse plants provided by Dr. R. Barker, originally from Dr. L. Brilman (*Lolium*), and Dr. M. Engelke (*Zoysia*). Tillers (2-3 cm) were rinsed with tap water for 30 min, surface sterilized in 15% bleach solution (0.79% sodium hypochlorite) and 0.1 ml/L of Tween-20 (Sigma Chemical Co., St. Louis, Mo) for 25 min, and rinsed three times in sterile water. Meristems (1.5 mm) were dissected from the tillers and placed in small glass tubes (10 x 150 mm) with 5 ml of initiation medium. Three wk later, shoots were transferred to multiplication medium. Plantlets were multiplied in GA7 Magenta boxes (Magenta Corp., Chicago, Ill) with a 2.5-wk transfer cycle. Growth-room conditions were 25°C with a 16-h light / 8-h dark photoperiod (cool white fluorescent illumination, 25 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Culture media

- Initiation medium: MS medium (17) with 3% sucrose, 0.2 mg/L kinetin (KT), 0.3% agar (Bitek agar, Difco, Detroit, Mich), and 0.1% Gelrite (Kelco, San Diego, Ca) adjusted to pH 5.8.
- Multiplication medium: MS medium with 0.5 mg/L KT for *Lolium* and 1.0 mg/L KT for *Zoysia*.
- Pretreatment medium for SF and VIT: Multiplication medium with 3.5% agar, 1.75% Gelrite and 5% DMSO.

- Recovery medium for SF and VIT: Multiplication medium (2 ml per well) in 24-well plates (Costar, Cambridge, Mass.).
- Recovery medium for E-D: Softer multiplication medium (agar 0.3% and Gelrite 0.05%) used to aid in rehydrating the beads.

Cold acclimation

Two wk after transfer to fresh medium, plantlets were cold acclimated (CA) for 4 wk in a growth chamber [-1°C 16 h dark/22°C 8 h with light (10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)] as the standard treatment (4, 21).

To determine the effects of CA on grass meristem cryopreservation, two-wk old plantlets of *Lolium perenne* cvs. Elka and Linn, *Z. matrellas* cv. DH 96-12, and *Z. japonicas* cv. Palisades were CA for 0, 1, 4, and 8 wk in the standard CA conditions. Two replications for each treatment were frozen (n = 40) with the slow freezing protocol (described below) and 5 controls were treated with cryoprotectant but not frozen.

Dehydration

The effect of air-dehydration on the moisture content and regrowth of encapsulated meristems was studied with 'Elka' and 'DH 96-12' based on the E-D procedure as described below. The encapsulated meristems were air-dehydrated for 2, 4, 5, 6, or 7 h under laminar flow and plunged into liquid nitrogen. Twenty meristems were cryopreserved in each treatment with two replications (n = 40). Five meristems were used for the non-frozen controls. Twenty meristems from each of the 10 cultivars were tested for their response to E-D with 6-h dehydration. Moisture content of beads with encapsulated meristems was determined for 30 beads dried under laminar flow for 0, 1, 2, 3, 4, 5, 6, and 7 h. Moisture content was determined as the difference between dry weight (DW) and fresh weight (FW) calculated as $[(\text{FW}-\text{DW})/\text{FW}] \times 100\%$. Dry weight was determined after heating the beads in an oven at 95°C for 24 h.

To determine the effect of duration of PVS2 dehydration on regrowth of meristems, 'Palisades' meristems were treated with PVS2 for 20, 35, 50, or 60 min and plunged into liquid nitrogen (described below). For each time period 20 meristems were vitrified and 5 controls were treated with PVS2, rinsed, and plated. The experiment was done twice (n = 40).

Cryopreservation procedures

Lolium perenne cvs. Elka and Linn, *Z. matrellas* cv. DH 96-12, and *Z. japonicas* cv. Palisades were tested with the following three techniques. Plantlets were cold acclimated (CA) for 4 wk (as noted above) before meristems (1 mm) were dissected for cryopreservation (4, 21).

Slow freezing (SF): The method used was originally developed for pear (22). Meristems from 4-wk CA plantlets were grown on pretreatment medium for 2 d under the CA conditions. Meristems were transferred to 0.25 ml liquid MS medium in 1.2 ml plastic cryovials (Cryovial, Beloeil, Quebec, Canada) on ice. One ml of the PGD cryoprotectant (10), a mixture with 10% each of polyethylene glycol (MW 8000), glucose and DMSO in MS liquid medium, was added drop wise over 30 min then equilibrated at 4°C for another 30 min. Samples were cooled in a programmable freezer at 0.1°C/min to -40°C (Cryomed, Forma Scientific, Mt., Clemens, Mich.) with ice initiation at -9°C, and plunged into liquid nitrogen. Meristems in the vials were thawed in 45°C water for 1 min, then 25°C water for 3 min, rinsed with liquid medium, and plated on recovery medium.

Encapsulation-dehydration: This method was originally developed for pear (7). Meristems from 4-wk CA plantlets were encapsulated in alginate beads (3% medium viscosity alginic acid (Sigma Chemical Co. St. Louis, MO) in liquid MS medium with 0.75 M sucrose and solidified in MS basal medium with 100 mM calcium chloride and 0.4 M sucrose). The beads were pretreated in liquid MS medium with 0.75 M sucrose for 18 h on a rotary shaker. Beads were removed from the pretreatment medium, placed on filter paper in sterile Petri dishes, and air dried under laminar flow for 4 h at 25°C (at about 40% relative humidity). Dried beads were transferred to 1.2 ml cryovials and plunged directly into liquid nitrogen. Vials were rewarmed at room temperature for 20 min and encapsulated meristems were transferred to recovery medium.

Vitrification: The technique used was developed for *Ribes* (14). Meristems from 4-wk CA plantlets were pretreated as for SF. Immediately before cooling the meristems were transferred to 1.2 ml cryovials with 1% bovine serum albumin (BSA) in 0.4 M sucrose MS liquid medium for 2 h. The BSA solution was replaced with PVS2 cryoprotectant (30% glycerol, 15% ethylene glycol and 15% DMSO in MS liquid medium with 0.4 M sucrose) (27) for 20 min, and vials were plunged directly into liquid nitrogen. Vials were rewarmed in 45°C water for 1 min, transferred to 25°C water for 2 min, rinsed in liquid medium with 1.2 M sucrose, and plated on recovery medium.

Data collection and statistical analysis

Regrowth data (new shoot growth) were taken after 3 wk on the recovery medium. Data were analyzed with ANOVA and Duncan's multiple range test with significance at $P \leq 0.05$ using STATGRAPHICS 7.0 (Statistical Graphics Corp. and STSC Inc., Rockville, MD, USA).

Results and discussion

Cold Acclimation

Cold acclimation was essential for successful SF cryopreservation of *Lolium* and *Zoysia* meristems. One wk CA significantly increased regrowth for the four genotypes tested and regrowth increased with CA duration except for 'Elka', which reached 100% recovery at 4 wk CA (Table 1). Without CA only 30% of the *Lolium* meristems regrew and all *Zoysia* meristems died. *Zoysia* cultivars required 8 wk CA for even moderate regrowth following SF.

Table 1. Recovery of *Lolium* and *Zoysia* meristems cold acclimated for 0 to 8 wk and cryopreserved by slow freezing.

Cultivar		Regrowth (%)			
		Weeks of cold acclimation			
		0	1	4	8
<i>Lolium</i>	Elka	33.3±11.7	85.0±7.1	100±0	100±0
	Linn	31.6±16.4	50.0±14.1	63.2±4.5	88.9±5.5
<i>Zoysia</i>	DH 96-12	0	6.3±1.83	20.0±0	30.0±7.1
	Palisades	0	5.0±0	15.0±7.1	50.0±21.2

Lolium, a very cold-hardy genus, is widely distributed in temperate regions. There is a considerable amount of literature showing low temperature treatment as one of the best strategies for cold acclimation of most temperate plants (3, 32). During cold acclimation many changes occur, cold-regulated genes are expressed, dehydrin or dehydrin-like proteins are produced (1, 2), and plasma membrane structure, cellular composition and cell wall components change (6, 33). Although *Zoysia* is a subtropical genus, it also cold acclimated under the alternating low temperature regime in our experiments. For both genera, cold acclimation significantly increased the regrowth of cryopreserved meristems (Table 1).

The response to CA obtained with *Lolium* and *Zoysia* is similar to results seen in *Rubus* and *Pyrus* cryopreservation where CA increased regrowth for subtropical as well as temperate species (21, 22). For some *Rubus* and *Pyrus* genotypes, one wk CA significantly increased the regrowth of meristems following cryopreservation, but some genotypes required much longer CA (4, 5).

The dehydration tolerance of alginate-encapsulated (but not cryopreserved) meristems was also improved by CA (Figure 1). CA meristems of 'Elka' and 'Palisades' retained high regrowth following dehydration for up to 7 hr, while 'DH 96-12' declined to 80%. All non-CA meristems survived a 2-h dehydration to 40% water content, but regrowth of all three genotypes declined sharply following 4-hr dehydration (22% water content) and dropped to almost zero with 6-h dehydration (17.6 % water content).

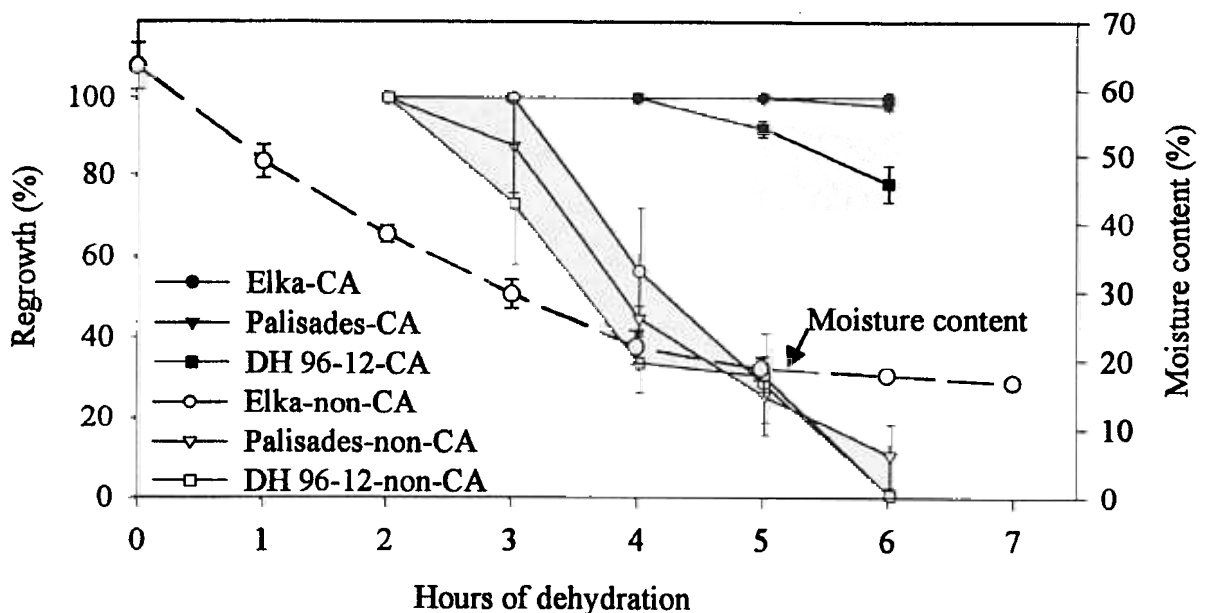


Figure 1. The effect of 4-week cold acclimation and moisture content on the regrowth of alginate encapsulated *L. perenne* cv. Elka, *Z. japonicas* cv. Palisades, and *Z. matrellas* cv. DH 96-12 meristems during air-dehydration (no LN).

Dehydration

Both *Lolium* and *Zoysia* responded well to E-D cryopreservation with 4-h to 7-h dehydration (Figures 2-3). The extent of dehydration was important for both genera. The standard 4-wk CA followed by a 6-h dehydration resulted in regrowth of all 'Elka' and 80-95% of 'Palisades' and 'DH 96-12' meristems. The largest improvement in recovery occurred with 4-h dehydration and gradually improved up to 7 h for 'Elka' (Figure 2) and 6 h for 'DH 96-12' (Figure 3). Control and cryopreserved 'Elka' meristems tolerated 7-h dehydration and moisture contents down to 16.5 % with little damage. Recovery of cryopreserved 'Elka' meristems was 80% with 4-h dehydration (22% moisture) and increased to nearly 100% with 7-h dehydration (16.5% moisture). Control 'DH 96-12' meristems (dried but not cryopreserved) had reduced regrowth after 4-h dehydration and recovery decreased to 60% at 7 hr. The cryopreserved 'DH 96-12' meristems had the best recovery with 5-h dehydration (19% moisture). Moisture contents of approximately 20% are successful for cryopreservation of several crops by E-D (9). Survival of mulberry and sugar cane shoot tips declined when encapsulated meristems were desiccated below 20% because of dehydration injury (12, 18).

High dehydration tolerance is a prerequisite for successful cryopreservation by encapsulation-dehydration because protoplasm must have low water content to vitrify, thus avoiding ice crystal formation when exposed to liquid nitrogen. However, low water content may cause dehydration damage to the cells, often in the form of membrane alterations. Cold acclimation increases dehydration tolerance by altering membrane composition (30). Cold acclimation also increases glass formation in woody plants (29). The best results for alginate-encapsulated pear meristems occur with about 20% bead-moisture content before plunging into liquid nitrogen (28). We also found that low bead-moisture content was necessary for successful grass cryopreservation. Cold-acclimated grass meristems tolerated as low as 16.5% bead-moisture content and had higher regrowth following E-D than non-acclimated meristems (Figure 1). Non-acclimated meristems could not tolerate long periods of dehydration and most died at bead-moisture contents of 25% or less. Scottez *et al.* demonstrated that the dehydration tolerance and regrowth of cold-acclimated pear shoot tips declined dramatically after only one day under deacclimating conditions and continued to lose dehydration tolerance at room temperature (28). Low-temperature treatment not only increased freezing tolerance but also increased the dehydration tolerance of grass meristems (Figures 1-3).

Screen of cryopreservation techniques

Slow freezing

Lolium meristem regrowth following SF was 90% for 'Elka' and 65% for 'Linn' (Table 2). *Zoysia* meristems had poor recovery and both 'DH 96-12' and 'Palisades' produced 15% or less regrowth. *Lolium*, a cold-tolerant temperate genus, responded well to slow freezing, but *Zoysia*, a subtropical genus, did not. The slow freezing method is based on freeze-induced dehydration. Extracellular ice formation increases intracellular dehydration resulting in a highly concentrated protoplasm that vitrifies on exposure to liquid nitrogen (26). After the 4-wk CA treatment all genotypes had increased freezing tolerance and improved regrowth following cryopreservation (Table 1).

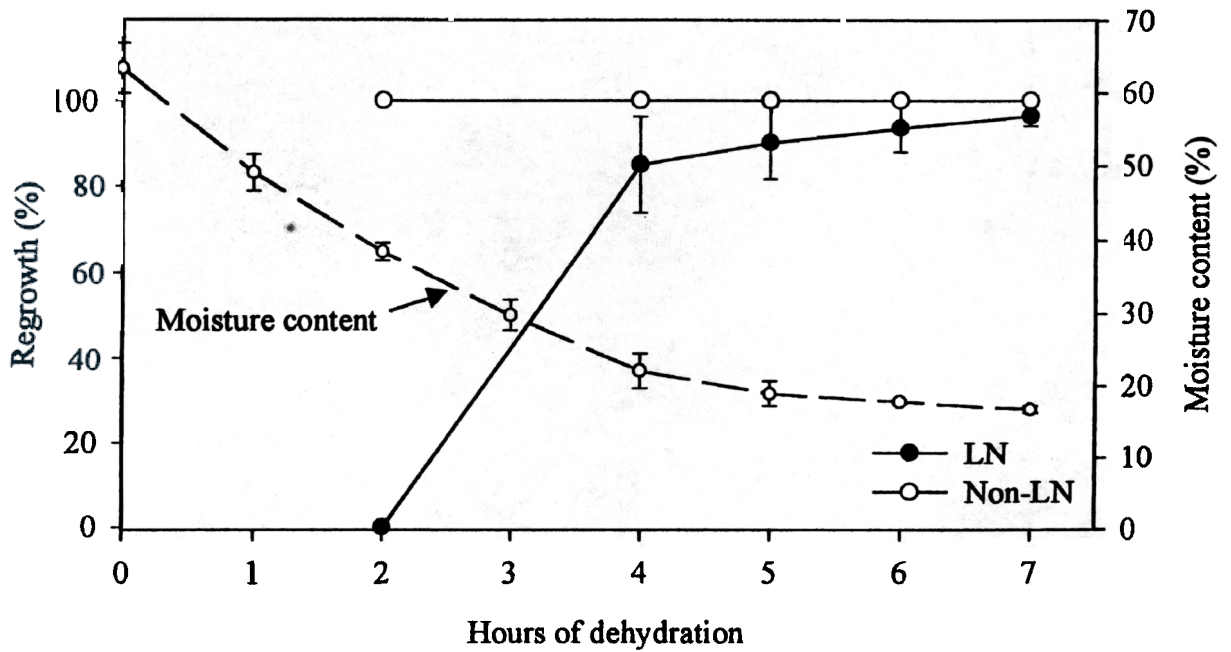


Figure 2. The effect of dehydration duration and moisture content on regrowth of *in vitro*-grown control and cryopreserved meristems of *L. perenne* cv. Elka meristems by encapsulation-dehydration. All materials were cold acclimated for 4 weeks before cryopreservation (LN).

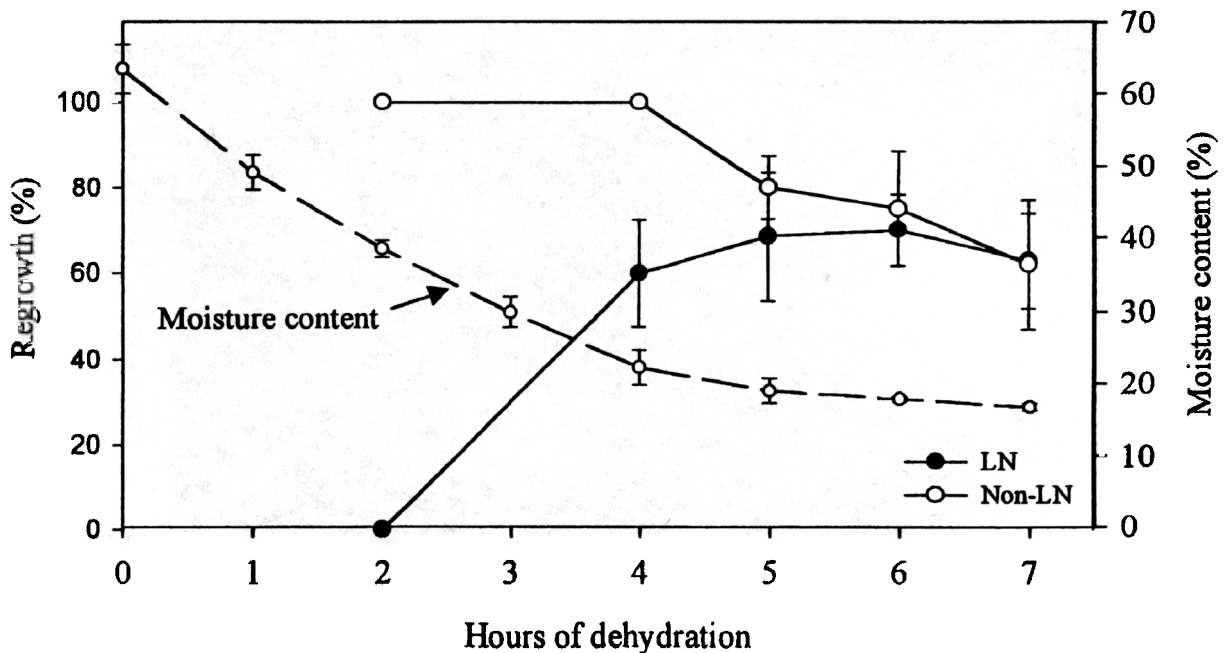


Figure 3. The effect of dehydration duration and moisture content on regrowth of *in vitro*-grown control and cryopreserved meristems of *Z. matrellas* cv. DH 96-12 meristems by encapsulation-dehydration. All materials were cold acclimated for 4 weeks before cryopreservation (LN).

Table 2. The regrowth of *Lolium* and *Zoysia* meristems following cryopreservation by standard methods of slow freezing, encapsulation-dehydration, or vitrification. All materials were cold acclimated for 4 wk before cryopreservation.

Genus/Cultivar		Regrowth (%)		
		Technique ^z		
		SF	E-D	VIT
<i>Lolium</i>	Elka	90±7.4 ^a	85±5.6 ^a	3.7±1.7 ^e
	Linn	65±12.7 ^b	45±3.5 ^c	15.5±2.6 ^d
<i>Zoysia</i>	DH 96-12	13.3±3 ^d	60±6.5 ^b	14.5±2.8 ^d
	Palisades	15±2.3 ^d	80±7.8 ^{ab}	5±2.5 ^e

^z SF: slow freezing. E – D: encapsulation-dehydration with 4-h air-dehydration. VIT: vitrification in PVS2 for 20 min. (n = 40). Means in a column with different letters are significantly different at P ≤ 0.05 by Duncans multiple range test.

Encapsulation-dehydration

The standard 4-hr dehydration resulted in moderate to high regrowth of all four cultivars (Table 2). Additional tests with 6-hr dehydration of the encapsulated meristems (based on Figures 2-3) produced excellent results (67-95%) for nine of the ten cultivars (Table 3). Cryopreserved meristems recovered quickly and regrowth was apparent within one day following reculture. There was no callus formation and full-sized plantlets were formed in about 2 wk. Plantlets were transplanted to the greenhouse with 100% survival. Only 'Diamond' meristems recovered poorly. We attributed the poor regrowth to hyperhydricity of the 'Diamond' plantlets in culture, a physiological disorder resulting in water soaking of the leaves. By improving growth conditions to eliminate hyperhydricity we improved meristem regrowth following E-D from 22% to 71%. Studies of other plants show that the physiological condition of meristem source plants is very important for successful cryopreservation (23, 35).

Table 3. Regrowth of 4-wk cold acclimated meristems of *Lolium* and *Zoysia* cultivars cryopreserved by encapsulation-dehydration after 6-h air dehydration (17.6 moisture content).

Cultivars		Regrowth (%)
<i>Lolium</i>	Elka	93
	Linn	67
	Mann	79
	SR	75
	4400	86
<i>Zoysia</i>	Cavalier	80
	DH 96-12	70
	Diamond	22 (71) ^z
	El Toro	91
	Palisades	95

^z Initial plants tested were hyperhydric /watersoaked. The second group (tested with improved growth conditions) was physiologically normal.

Vitrification

None of the four cultivars tested responded well to the vitrification procedure used, and regrowth for all four was below 16% (Table 2). Additional tests indicated that the 20-min dehydration with PVS2 was not adequate for vitrification of grass meristems. Increased duration of PVS2 exposure improved regrowth of 'Palisades' meristems from 5% to 40%, but the longer exposure to PVS2 killed 60% of the control meristems (Figure 4). This indicates the possibility of further improving vitrification results through additional CA and longer PVS2 exposure.

PVS2 solutions are toxic to some plants but the toxicity can sometimes be decreased with pretreatments employing bovine serum albumen (BSA), proline, or glycerol and sucrose solutions (14, 26). It may be necessary to use an alternative vitrification solution with the grass meristems. Several crops that show PVS2 toxicity respond well to PVS3 (50% (w/v) glycerol and 50% (w/v) sucrose in basal medium (15, 19, 35).

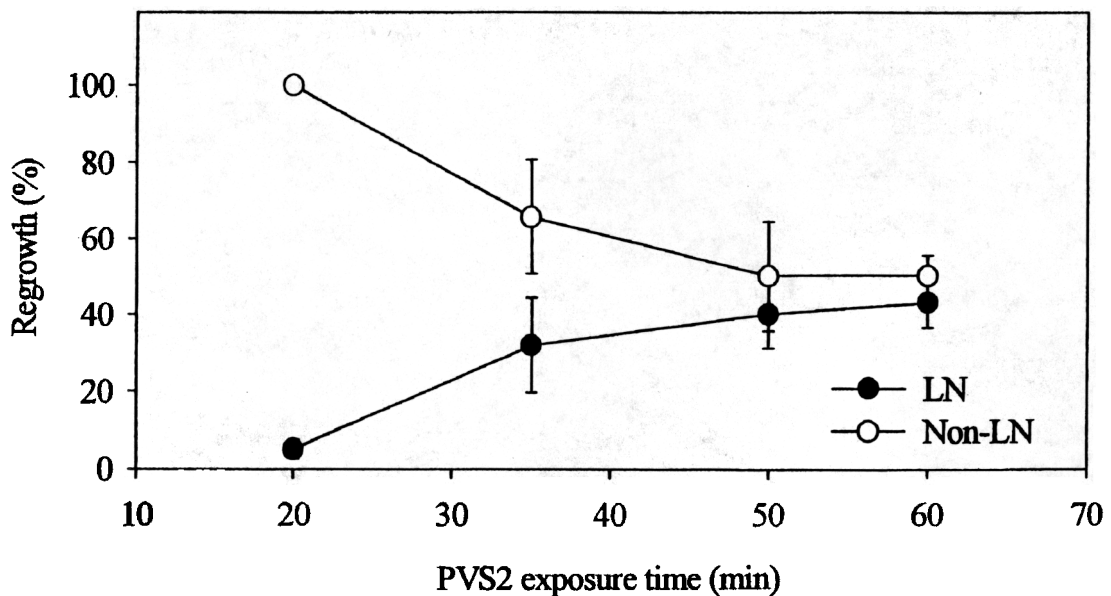


Figure 4. The effects of exposure time to PVS2 at 0°C on the regrowth of *Z. japonicas* cv. Palisades meristems following cryopreservation by vitrification. All materials were cold acclimated for 4 weeks before cryopreservation (LN).

Conclusions

Cold acclimation was essential for successful cryopreservation of both *Lolium* and *Zoysia* meristems. Cold acclimation not only increased freezing tolerance but also significantly increased meristem dehydration tolerance. Cold-acclimated meristems tolerated dehydration to 16.5% - 22% water content and they responded to the stress with improved recovery from liquid nitrogen. Cryopreservation by slow freezing and encapsulation-dehydration produced high regrowth for the five temperate *Lolium* cultivars. The five subtropical *Zoysia* cultivars produced excellent regrowth following encapsulation-dehydration. One hundred meristems of each of these cultivars were stored in liquid nitrogen as part of the U.S. National Plant Germplasm Collection.

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