

## A COMPARATIVE STUDY OF THREE CRYOPRESERVATION PROTOCOLS FOR EFFECTIVE STORAGE OF *IN VITRO*-GROWN MINT (*Mentha SPP.*)

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### Abstract

This study was designed to determine the response of diverse mint genotypes to three commonly used cryopreservation techniques. Four mints [*Mentha x piperita* nothosubsp. *citrata* (Ehrh.) Briq.; *M. canadensis* L.; *M. australis* R. Br, and *M. cunninghamii* Benth] were cryopreserved using three protocols: controlled rate cooling (CC), encapsulation dehydration (ED) and PVS2 vitrification (VIT). Regrowth of mint species following controlled rate cooling (93%) was significantly ( $P < 0.0001$ ) better than encapsulation dehydration (71%) and vitrification (73%). All four genotypes responded well to the controlled rate cooling protocol but there was some variability with the other two protocols. Genotype specific response to the individual protocols showed that there were significant differences in the recovery of *Mentha x piperita* nothosubsp. *citrata* and *M. australis* with  $CC > VIT > ED$ . There were also significant differences in the recovery of *M. cunninghamii* and *M. canadensis*, with CC and ED significantly better than VIT. Regrowth of the shoot tips of these mints ranged from 60% to 95% for all but one treatment. The overall results of this study compare favorably to other techniques. These improved results may be due to a combination of favorable growth conditions, cold acclimation and recovery medium. Controlled rate cooling was the most successful technique for the storage of these diverse mint genotypes; however recovery of shoot tips from VIT and ED was high and these techniques could also be used for cryogenic storage of mint germplasm.

**Keywords:** cold acclimation, controlled rate cooling, encapsulation dehydration, *in vitro*, vitrification

### INTRODUCTION

Cryopreservation has developed greatly in the last 10 years and is now a reliable option for the long-term storage of clonal selections (6, 19). Diverse genotypes can be cryopreserved, but several techniques may be required to store large collections due to differential genotypic response (25). Optimal results from cryopreservation require a combination of good plant

growth conditions, adequate conditioning of plant materials, properly performed protocols, and good regrowth conditions. Cryopreservation protocols that are effective for diverse species or cultivars are needed for conservation programs and genebanking, and sometimes available techniques can be directly applied to new species (17).

Mint (*Mentha* spp.) belongs to the family Lamiaceae which includes over 2500 species and varieties (1). Seeds can be used to preserve some types of mint, but sterile species and clonal selections require an alternative option to retain the unique characteristics of the genotype and guard against loss. Cryopreservation protocols that achieve high recovery are available for many individual *Mentha* species. Towill (27) found 29 to 100% survival of mint shoot tips following a controlled cooling procedure with a cooling rate of 0.25 °C/min to -35°C followed by plunging in liquid nitrogen (LN). Towill (28) also provided the first report on vitrification of *Mentha* with a mean recovery of 44% (*M. aquatica* L. x *M. spicata* L.). Towill and Bonnart (29) examined shoot tips of mint species (*M. aquatica*, *M. arvensis*, *M. piperita* and *M. spicata*) following extensive physical cracking of the external glass within semen straws during vitrification and found that this condition did not adversely affect survival. Hirai and Sakai (7) achieved 90% recovery of *Mentha spicata* (spearmint) using encapsulation vitrification. This compared to 50% recovery with encapsulation dehydration in the same study and with 51.7% recovery reported by Sakai et al. (22). Senula et al. (24) recovered up to 89% of *Mentha x piperita* L., *M. x villosa* Huds and *M. spicata* L using droplet vitrification. Forty-six core accessions, representing over 20 *Mentha* species were screened for cryopreservation at the National Center for Genetic Resources Preservation, Fort Collins, CO (25). Thirty-one accessions (67%) of these were successfully cryopreserved following a sucrose pretreatment and PVS2 vitrification on aluminum foil. The remaining accessions were cold acclimated for 6 weeks, and 8 accessions were successfully cryopreserved using PVS2 vitrification in tubes and the five accessions remaining were successfully cryopreserved using encapsulation dehydration. These results are based on a standard of 40% minimum viability, that was shown to provide adequate recovery from stored samples (16).

Although there seem to be many successful cryopreservation programs already in place for mint storage, often results are not effective for all species. There are also no well controlled comparisons of these commonly used protocols. The objective of this study was to directly compare three well-tested techniques, controlled rate cooling, PVS2 vitrification and encapsulation dehydration, and to determine their efficiency as storage protocols for diverse mint genotypes.

## MATERIALS AND METHODS

### *Plant material*

Micropropagated *Mentha* spp. [*Mentha x piperita* nothosubsp. *citrata* (Local # 124.001;PI 557993); *M. canadensis*, (571.001; PI 557613); *M. australis* (690.001;PI 617498) and *M. cunninghamii* (666.001; PI 617481)] from the *in vitro* collections of the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR, were multiplied on NCGR-Mentha (MEN) medium containing MS mineral salts and vitamins (11), with 0.5 mg·l<sup>-1</sup> N<sup>6</sup>benzyladenine (BA), 0.1 mg·l<sup>-1</sup> indole-3-butyric acid (IBA), 3.0 g/l agar (Difco, Detroit, MI), 1.25 g/l gelrite (PhytoTechnology Lab., Shawnee Mission, KS), and 30 g/l sucrose at pH 5.7, dispensed in Magenta GA<sub>7</sub> boxes (Magenta Corp., Chicago, IL). The plantlets were subcultured every 3 weeks and grown at 25 °C under a 16 h light and 8 h dark photoperiod (40 μE·m<sup>-2</sup>·s<sup>-1</sup>).

### *Cold acclimation (CA)*

Plantlets three weeks after the last subculture were cold acclimated for 2 weeks with alternating cold and warm temperatures [22°C 8-h light ( $10\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and -1°C 16-h dark]. Shoot-tips (approx. 1 mm) were excised from cold-acclimated plantlets.

### *Encapsulation-dehydration procedure(ED)*

The protocol of Dereuddre (5, 4), as described by Chang et al (2) was used for this study. Two-week CA shoot tips were excised and placed in 3% (w/v) low viscosity alginic acid (Sigma, USA) in liquid MS without calcium at pH 5.7. Beads were formed by suspending shoot tips in alginate and dripping them into a saturated calcium chloride solution (MS medium with 100 mM  $\text{CaCl}_2$  and 0.4M sucrose). Beads were allowed to polymerize for 20 min. Encapsulated shoot tips were preconditioned in liquid MS with 0.75 M sucrose on a rotary shaker (50 rpm) for 18 h. Beads were blotted dry on sterile filter paper, transferred to an open glass Petri dish and dehydrated for 5.5 hrs (approx. 36% bead residual moisture content) in a laminar-flow hood at 0.6 m/sec air flow with ambient temperature of ~22°C and  $35\pm 2\%$  relative humidity. Dehydrated beads (20 per treatment) were placed in 1.2 ml cryovials (10 beads per cryovial) and plunged into liquid nitrogen (LN). The cryovials were rewarmed in 45°C water for 1 min and in 25°C for 1 min. The beads were rehydrated in liquid MS medium for 5 min before transfer onto MEN regrowth medium (0.5  $\text{mg}\cdot\text{l}^{-1}$  BA, 2.5 g/l agar, 1 g/l gelrite, and no IBA) in 24-cell tissue culture plates (Costar, Cambridge, MA). Pretreatment controls were set to determine that shoot tips remained viable after each critical step by plating 5 beads each after: 1) 18 h preconditioning in liquid MS medium with 0.75 M sucrose and 2) following air dehydration under the laminar flow hood.

### *Water content determination*

Four sets of 10 empty dehydrated beads were used to determine the moisture content [(Fresh Wt - Dry Wt)/Fresh Wt x 100]. Fresh weight is the weight of beads after dehydration under the flow hood. Dry weight is the oven dried (~103°C, 12 h) weight of the same beads.

### *Vitrification procedure (VIT)*

The PVS2 vitrification procedure described by Yamada et al. [31] and modified by Luo and Reed (9) was used. Twenty-five shoot-tip explants from 2-wk CA *Mentha* plantlets were pretreated for 48 h on MS agar plates containing 5% (v/v) dimethyl sulfoxide [DMSO, Sigma-Aldrich Co., St Louis, MO] with 3.5 g agar and 1.75 g/l gelrite under CA conditions. Shoot tips were transferred into 1.2 ml cryovials and treated with 1 ml loading solution (LS) [2 M glycerol in 0.4 M sucrose MS medium (v/v), pH 5.8] for 20 min at 25 °C. LS was removed and 1 ml PVS2 cryoprotectant solution (30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose) (20) was added at 25 °C and held for 20 min. Five shoots of each accession were rinsed in liquid MS medium containing 1.2 M sucrose and planted in regrowth medium as described above to determine sensitivity to PVS2. Twenty shoots of each accession in PVS2 solution were plunged into LN for each replication. Rewarming was done as described above for encapsulation dehydration. Shoot tips were plated on MEN regrowth medium.

### *Controlled rate cooling (CC)*

The controlled rate cooling protocol used was described by Reed (14, 19). Shoot-tips from 2 wk CA *Mentha* plantlets were excised and placed on 5% DMSO agar plates as above for 48 h in CA conditions. Shoot tips were transferred to 1.2 ml plastic cryovials on ice containing 2 drops of liquid MS medium. Two drops of the PGD cryoprotectant [10% each polyethylene glycol (MW 8000), glucose, and DMSO in liquid MS medium] were added to

cryovials every 2 min for 6 min then 4 drops every 2 min over a 30 min period. Cryovials were equilibrated for 30 min on ice. Excess cryoprotectant was removed to 1 ml before loading samples into a programmable freezer (Cryomed, Leona, MI), (0.1°C/min to -9°C for seeding, to -40°C). At the end of the programmed run, the samples were plunged in LN. Samples were rewarmed as above, rinsed in liquid MS medium and plated on MEN regrowth medium. The unfrozen controls were left on ice until the cooled samples exhibited an exotherm, after which control shoot tips were rinsed in liquid MS and plated on MEN regrowth medium and the cooled samples continued cooling in the freezer.

#### *Experimental design and data analysis*

Regrowth assessment was done 6 weeks after rewarming. Greening, shoot production and leaf expansion were all required for samples to qualify as fully recovered from cryopreservation. Each experiment included 20 cryopreserved and 5 to 10 unfrozen shoot tips per treatment and was replicated three times. Data were analyzed by analysis of variance (ANOVA). The data are presented as percentages with means separation using Duncan's Multiple Range Test (Alpha = 0.05) (23).

## RESULTS

All of the mint accessions cryopreserved with the three protocols produced moderate to high regrowth after liquid nitrogen exposure. Shoots developed without intermediate callus formation. ANOVA showed a significant ( $P < 0.0001$ ) interaction of genotype and technique (Table 1).

Table 1. Factorial ANOVA of Genotype by Treatment Interaction

	DF	Mean square	<i>Pr</i> > <i>F</i>
Genotype	3	37.2615	< 0.0001
Technique	2	67.2314	< 0.0001
Genotype * Technique	6	33.5407	< 0.0001
Error	24	1.05555	

#### *Comparison by Technique*

When data for all four accessions were pooled, the regrowth following the CC protocol (93%) was significantly better ( $P < 0.0001$ ) than recovery with the other two techniques. Mean regrowth of shoot tips following encapsulation dehydration (71%) and vitrification (73%) did not differ significantly.

### Response of genotypes to cryopreservation protocols

For *Mentha x piperita* nothosubsp. *citrata*, each technique produced significantly different recovery percentages ( $P < 0.0001$ ). The best recovery was with CC and yielded 95% regrowth, followed by VIT with 60% and ED with about 40% (Fig. 1A). *M. australis* recovery also varied significantly among the protocols, but all were above 76% (Fig. 1B). *M. cunninghamii* had good recovery with CC and ED ( $> 80%$ ); however, recovery was significantly less with VIT (61%) (Fig. 1C). *M. canadensis* recovery was not significantly different between CC and ED ( $> 90%$ ) but regrowth following the VIT protocol was significantly less, at 78% (Fig. 1D).

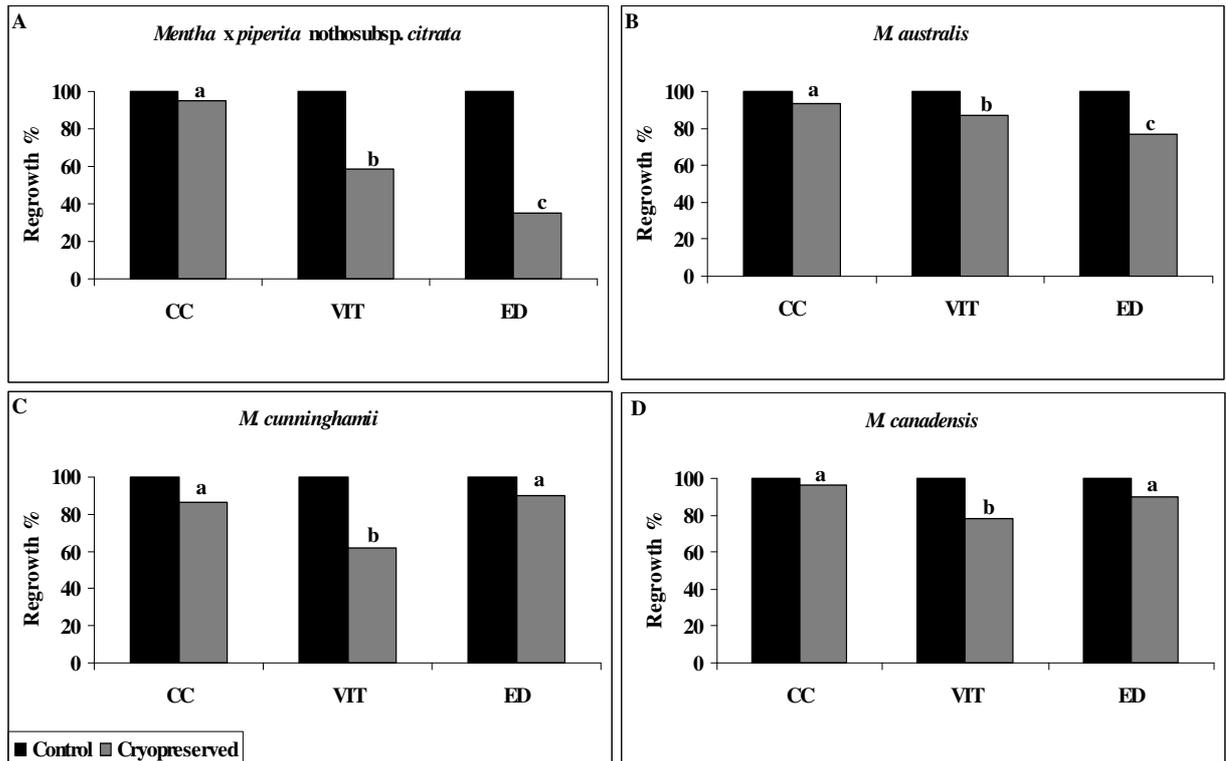


Fig. 1: Effect of cryopreservation protocols on the regrowth of cryopreserved *Mentha* species. Shoot tips were cryopreserved with Controlled rate cooling (CC), Encapsulation dehydration (ED) or PVS2 vitrification (VIT). Means with the same letter are not significantly different at  $\alpha = 0.05$ .

## DISCUSSION

Developing a new cryopreservation method for every plant accession is difficult, time consuming, and makes backing up large germplasm collections very difficult. With the continued development of cryopreservation techniques, it is now possible to choose a technique and quickly adapt it to a new species (19). Application of tested methods to related plant types can considerably speed up storage of plant collections (17). In the case of mint, all three of the standard NCGR cryopreservation techniques produced good to excellent regrowth following LN exposure.

Cold acclimation and CC is a new method for mint cryopreservation and it produced the best overall recovery for all genotypes (Fig 1). Comparison of the three protocols showed significantly better recovery ( $P < 0.0001$ ) using the CC protocol compared to the other two techniques. Regrowth of the four mint species following controlled rate cooling (93%) was significantly ( $P < 0.0001$ ) better than encapsulation dehydration (71%) and vitrification (73%). Our results are similar to studies of other temperate plants cryopreserved by CC following cold acclimation (14, 17, 15). These results show that the CC protocol would be useful for the cryopreservation of difficult-to-cryopreserve mint accessions as reported by Staats et al. (25). The ED and VIT techniques were more variable, but produced moderate to good results for all genotypes.

*M. piperita* nothosubsp. *citrata* had 95% recovery from controlled rate cooling (Fig. 1A) compared to the 71% regrowth reported by Towill (27). Differences in plant culture conditions, pretreatments, cooling rates and intermediate cooling temperatures can affect recovery and may account for the observed difference. Towill used 6 to 8 wk culture without CA pretreatment and a 0.25°C/min cooling rate to a pre-plunge temperature of -35°C while we used CA plants with 0.1°C/min to -40 °C (27).

The PVS2 vitrification protocol is routinely used for successful cryopreservation of both temperate and tropical crop species (3, 12, 21, 26). However, most plants are sensitive to the highly concentrated PVS2 solution, even for a short duration (10, 13, 30). Thus there is a need to carefully regulate the exposure time for each accession. This requirement for critical timing can make it difficult to handle many samples at the same time. Several VIT protocols are successfully used to cryopreserve mint. Towill [28] found recovery of 44 % from mint shoot tips (*M. aquatica* x *M. spicata*) using a vitrification solution of 35% ethylene glycol, 1 M DMSO, 10% PEG (MW 8000). Staats et al. (25) used two VIT techniques for storing a group of mint accessions. The PVS2 droplet technique with sucrose pretreatment was successful for 31 of the 46 mint accessions tested, including three included in our study. Eight that did not respond to droplet vitrification did recover well from CA and standard PVS2 vitrification. Senula et al. [24] tested cryopreservation of six mint accessions using PVS2 droplets on aluminum foil. Recovery following LN exposure improved significantly when source plants had 2 to 6 wks alternating temperature CA of 25°C/-1°C, similar to the CA protocol used at NCGR. They found that with alternating temperature CA *M. spicata* recovery significantly improved from 68 to 98%, *M. x villosa* from 60 to 70% and *M. x piperita* from 57 to 80%.

Recovery of shoot tips following ED was not significantly different from VIT in our study. Three of the four mint accessions in our study had good recovery following ED (> 75%), with only *M. x piperita* nothosubsp. *citrata* having low regrowth (~40%) (Fig. 1A). Sakai et al. (22) and Hirai and Sakai (7) found that apices of *M. spicata* cv. spearmint recovered at 51.7% when cryopreserved with the conventional encapsulation-dehydration protocol but regrowth increased to 87% with encapsulation-vitrification protocol. However, for Staats et al. ED was successful for five of eight accessions that did not respond well to two VIT protocols (25).

Recovery of plants following cryopreservation is dependent on a variety of factors including the age of the mother plants, pretreatments, the length and type of CA, the technique used and the recovery process. Variation in results can arise from any of these factors. Callus formation in plants is often promoted in the presence of auxin (8). Injury which occurs during cryopreservation promotes callus formation, but removing IBA from the recovery medium significantly decreased callus proliferation in cryopreserved blackberries (2). The stock plant materials used for this study were grown on medium containing plant growth regulators (BA and IBA), but we excluded IBA (auxin) from the cryopreservation recovery medium. Towill

(27) observed callus formation on mint shoot tips cryopreserved by slow cooling while the mints in our study did not form callus.

Differential results can also occur from subtle changes in the growth of the mother plants or in the composition of the recovery medium. Optimal results from cryopreservation require a combination of good plant growth conditions, adequate conditioning of plant materials, properly performed protocols, and good regrowth conditions. Differences in the standard growth protocols in the mint studies cited above may have important implications in the recovery of plants using any of the standard techniques. For example our standard protocol for mint includes PGRs in the growth medium but removes auxin from the cryopreservation recovery medium, while Staats et al. (25) used PGR-free medium for the mother plants and applied PGRs for regrowth. Standardization of these culture variables could be an important key to the variability noted between laboratories using the same techniques and plant materials (18).

This comparison of three cryopreservation protocols found that all the procedures were more than adequate for storage of mint collections. Recovery of shoot tips was 60% to 95% for the four genotypes, much better than the 40% minimum required for secure long-term storage of germplasm collections (16, 17). These results indicate that the choice of technique may be less important than the care of the plant material and the careful execution of the protocol. The overall results of this study compare favorably to other studies using a variety of techniques.

The high regrowth noted in our study is likely due to the combination of favorable growth conditions for the mother plants and suitable cold acclimation before cryopreservation, as well as regrowth on an optimal recovery medium. Overall, for cryopreservation of mint germplasm we recommend cold acclimation of healthy *in vitro*-grown shoot cultures, followed by carefully executed standard cryopreservation techniques, and recovery on medium without auxins.

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