

CRYOPRESERVATION OF *IN VITRO*-GROWN GOOSEBERRY AND CURRANT MERISTEMS

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SUMMARY

Apical meristems from in vitro-grown plants of currant and gooseberry (*Ribes*) germplasm were successfully cryopreserved using a variety of techniques. Modifications of slow freezing, vitrification and encapsulation-dehydration were compared. Slow freezing at 0.3 or 0.5°C/min following pregrowth on 5% DMSO and cryoprotection with PGD produced moderate to high survival. Vitrification in PVS2 following pregrowth on sorbitol and a 20-minute pretreatment resulted in low to moderate survival, while pregrowth on 5% DMSO improved survival for two of the three genotypes tested. Modification of the vitrification procedure with a pretreatment similar to that used in slow freezing was not effective. Encapsulation-dehydration with an 18-hour preculture with 0.75M sucrose and 3 hrs of dehydration was very effective.

KEY WORDS: Cryopreservation, *Ribes*, currants, gooseberries, vitrification, encapsulation-dehydration, slow-freezing.

INTRODUCTION

Cryopreservation as a form of germplasm preservation is a rapidly changing discipline. Many new techniques and modifications of old techniques are available for cryopreservation of plant materials. Slow freezing (two-step freezing) has many variations and its success depends on factors ranging from genotype to cooling rate (1,2,3,4). Vitrification relies on pretreatment with viscous cryoprotectants and has developed with many variations (5,6,7). The encapsulation-dehydration technique dehydrates meristems by both osmotic and evaporative methods and has been applied to apices of several plant species (8).

Few *Ribes* species and cultivars have been evaluated for their ability to survive cryopreservation. Sakai and Nishiyama (9) obtained excellent results in testing dormant

bud material of *Ribes grossularia* L. cv. Oregon Champion gooseberry and *R. rubrum* L. cv. London Market red currant for survival following exposure to liquid nitrogen but actively growing tissues have not been studied. Knowing the efficacy of available cryopreservation methods on several members of a genus could provide a basis for choosing one technique for routine germplasm storage. This study compares slow freezing, vitrification and alginate-bead dehydration methods using meristems of three genotypes of actively growing in vitro-cultured *Ribes*.

MATERIALS AND METHODS

General growth conditions: Micropropagated plantlets were multiplied and meristems recovered on NCGR-*Ribes* medium (RIB), which contains the mineral salts and vitamins of Murashige and Skoog (10) but with only 30% of the normal ammonium and potassium nitrate concentrations, and (per liter): 50 mg ascorbic acid, 20 g glucose, 0.1 mg N⁶-benzyladenine, 0.2 mg GA₃, 3.5 g agar (Bitec, Difco, Detroit, MI) and 1.45 g Gelrite (Kelco, San Diego, CA) at pH 5.7. Plants were grown at 25°C with 16-hr days (25 μmol·m⁻²·s⁻¹). All cultures were cold acclimated for one week in an incubator (8 hr days at 22°C and 16 hr nights at -1°C) before 0.8 mm meristems were excised (2).

Plant materials: Genotypes used were: *R. aureum* Pursh, currant, wild collection from northern Idaho, USA; *R. diacantha* Pall., gooseberry, wild collection from Tselinograd, Kazakhstan; *R. rubrum* L. cv. Cherry, red currant from Italy.

Slow freezing: The method used was developed for pear (2). Meristems were acclimated in the incubator (see general growth conditions) for two days on RIB medium with 5% DMSO and additional Gelrite (0.3 g/l) in the medium, then transferred to 0.25 ml liquid RIB medium in 1.2 ml plastic cryotubes and 1 ml of the cryoprotectant PGD (10% each polyethylene glycol (MW 8000), glucose and DMSO in RIB liquid medium) was added over 30 min. A 30 min equilibration at 4°C was followed by cooling at 0.3 or 0.5°C/min to -40°C and plunging in LN. Samples were thawed for one min in 45°C water then transferred to 22°C water for 2 min, rinsed in liquid RIB medium and plated on RIB medium for recovery.

Encapsulation-dehydration: The method used was developed for pear (8). Meristems were dissected onto agar plates then encased in alginate beads (3% low viscosity alginic acid with 0.75 M sucrose) for 18-hr pretreatment in liquid RIB medium with 0.75 M sucrose. Following pretreatment, beads were separated out on sterile petri dishes and air dried in the laminar flow hood for 2 or 3 hours, placed in cryotubes and plunged into LN. Vials were rewarmed at room temperature for 15 min then encapsulated meristems were planted on RIB recovery medium. For moisture content determination, beads were weighed, dried in an oven at 104°C for 16 hr and reweighed.

Vitrification: A modification of the technique for white clover was used (11). Meristems were pretreated for two days in the incubator (see general growth conditions) on RIB medium with either 5% DMSO and additional Gelrite or with 1.2 M sorbitol. PVS2 cryoprotectant (30% glycerol, 15% ethylene glycol and 15% DMSO in liquid RIB medium with 0.4 M sucrose) was added to cryotubes on ice and meristems were added and stirred. After 20 min, vials were submerged in LN. Samples were rewarmed for one min in 45°C water then transferred to 22°C water for 2 min and rinsed in liquid RIB medium with 1.2 M sucrose.

PGD-PVS2: A modification of the vitrification method was tested for improved survival of control and cooled meristems after exposure to PVS2. Meristems pretreated for 2 days on 5% DMSO medium were treated with the cryoprotectant PGD (30 min addition as in slow freezing) before exposure to PVS2 for 20 min. Cooling and rewarming were the same as the vitrification procedure.

Data analysis: Survival was measured as the number of green, growing plantlets six weeks after rewarming. Each experiment was done five times for a total of 100 meristems per genotype per trial. Analysis of variance and means separation were applied to the data using MSTATC software (Michigan State University). Significance is stated, at $P \leq 0.05$.

RESULTS AND DISCUSSION

Genotypes

***R. rubrum* cv. Cherry:** Only meristems frozen using the encapsulation-dehydration method with a 3-hr dehydration period recovered at a level similar to the cryoprotectant-treated controls (Figure 1A). Survival of meristems with all other cooling techniques was not significantly different between treatments but was significantly less than the controls. Significantly fewer controls of the vitrification (sorbitol) treatment survived compared to controls of the slow freezing techniques. Highly concentrated vitrification solutions were shown to have harmful effects on meristems of other species due to osmotic stress and chemical toxicity (12). *R. rubrum* cv. Cherry was sensitive to changes in culture conditions (data not shown), and the low survival following cryopreservation may reflect sensitivity to dehydration, pretreatments or cryoprotectants. Additional pretreatments such as preculture with 0.4M sucrose may be needed to further condition more sensitive genotypes prior to cooling (13).

R. diacantha: Cryopreserved meristems had moderate to high survival with all seven techniques tested and were not significantly different from the cryoprotectant-treated controls except for the 2-h encapsulation-dehydration and PGD-PVS2 (Figure 1B). Slow freezing (0.5°C/min) and vitrification (DMSO) techniques were significantly better for cryopreserving *R. diacantha* meristems than the PGD-PVS2 treatment, but not significantly different from the other techniques.

R. aureum: Survival of control and cryopreserved meristems was not significantly different among treatments except the 0.5°C/min slow freezing and the 2-hr encapsulation-dehydration methods (Figure 1C). The least variation between control and cryopreserved meristems was with the 3-hr encapsulation-dehydration treatment. Vitrification controls of *R. aureum* grown for two days on sorbitol pretreatment plates and treated for 20 min with PVS2 cryoprotectant had significantly lower survival than controls for the other freezing techniques. This low survival indicated an intolerance to sorbitol by *R. aureum*, since DMSO pretreated controls treated for 20 min with PVS2 retained high survival. Additional experiments with growth of *R. aureum* meristems on medium with and without 1.2 M sorbitol (not cooled) showed definite growth inhibition followed by death (100%) of those on sorbitol medium while 90% of those on regular medium survived and grew. Sorbitol in the medium improved survival of cryopreserved meristems of white clover (14) but was not favorable to the growth of *R. aureum* and possibly other *Ribes* genotypes.

Techniques

Slow freezing: Slow freezing resulted in low to good survival of meristems and there were no significant differences in survival for any of the genotypes between meristems cooled with the two freezing rates tested (Figure 1). These results differ from those for pear (2) and *Vaccinium* (15) where survival of meristems frozen at 0.3°C/min was significantly better than those frozen at 0.5°C/min. Additional osmotic pretreatments may improve survival by increasing cell dehydration before cooling begins. An alternative would be the use of slower cooling rates to allow additional dehydration.

Encapsulation-dehydration: Three-hr dehydration (39% moisture) of alginate-encapsulated meristems produced significantly higher survival than did 2 hr dehydration (50% moisture) for *R. aureum* and 'Cherry'. Additional dehydration to lower moisture levels (20% in pear) may further improve survival (16).

Vitrification: Survival of meristems from vitrification varied with genotype, and low survival was not alleviated by the additional pretreatment with PGD solution before exposure to PVS2. Pretreatment with DMSO for 2 days was equal to or better than sorbitol pretreatment for 2 of the 3 *Ribes* genotypes due to the toxic effects of sorbitol on some genotypes. Niino et al. (5) showed that genotypes respond differently to exposure to vitrification solutions. Additional pretreatments, such as incubation in highly osmotic sucrose solutions to condition the meristems before exposure to vitrification solutions, may overcome both the low survival and the variability within treatments.

CONCLUSIONS

Meristems from in-vitro grown plants of three *Ribes* species were successfully cryopreserved. Overall, the encapsulation-dehydration method was the most successful, but each genotype responded differently to the techniques. A genotype such as *R. diacantha* could be preserved with most of the methods tested while *R. aureum* is restricted to fewer successful techniques and *R. rubrum* cv Cherry survived well only with 3-hr encapsulation-dehydration treatment.

Each method has distinct advantages and disadvantages. Slow freezing was effective for many taxa but requires a special freezing apparatus, which might limit its use. Encapsulation-dehydration worked well for all the genotypes tested and does not require controlled warming or cryoprotectants, but beads must be handled individually and thus this method is more time consuming and tedious than the other methods. Vitrification is quick, however, the solutions tend to be toxic to the plants and careful timing is necessary for successful recovery of controls as well as cryopreserved meristems. The use of PGD as a pretreatment to minimize the toxicity of PVS2 did not improve survival following vitrification.

The availability of many techniques, each successful for a particular genus or species, allows more flexibility for scientists whether they are looking to conserve the range of genetic diversity or to preserve a few selected cell lines. The results of this study indicate the importance of screening several techniques when a range of genotypes will be stored. Variability in survival due to genotype differences can be overcome by choosing an appropriate method.

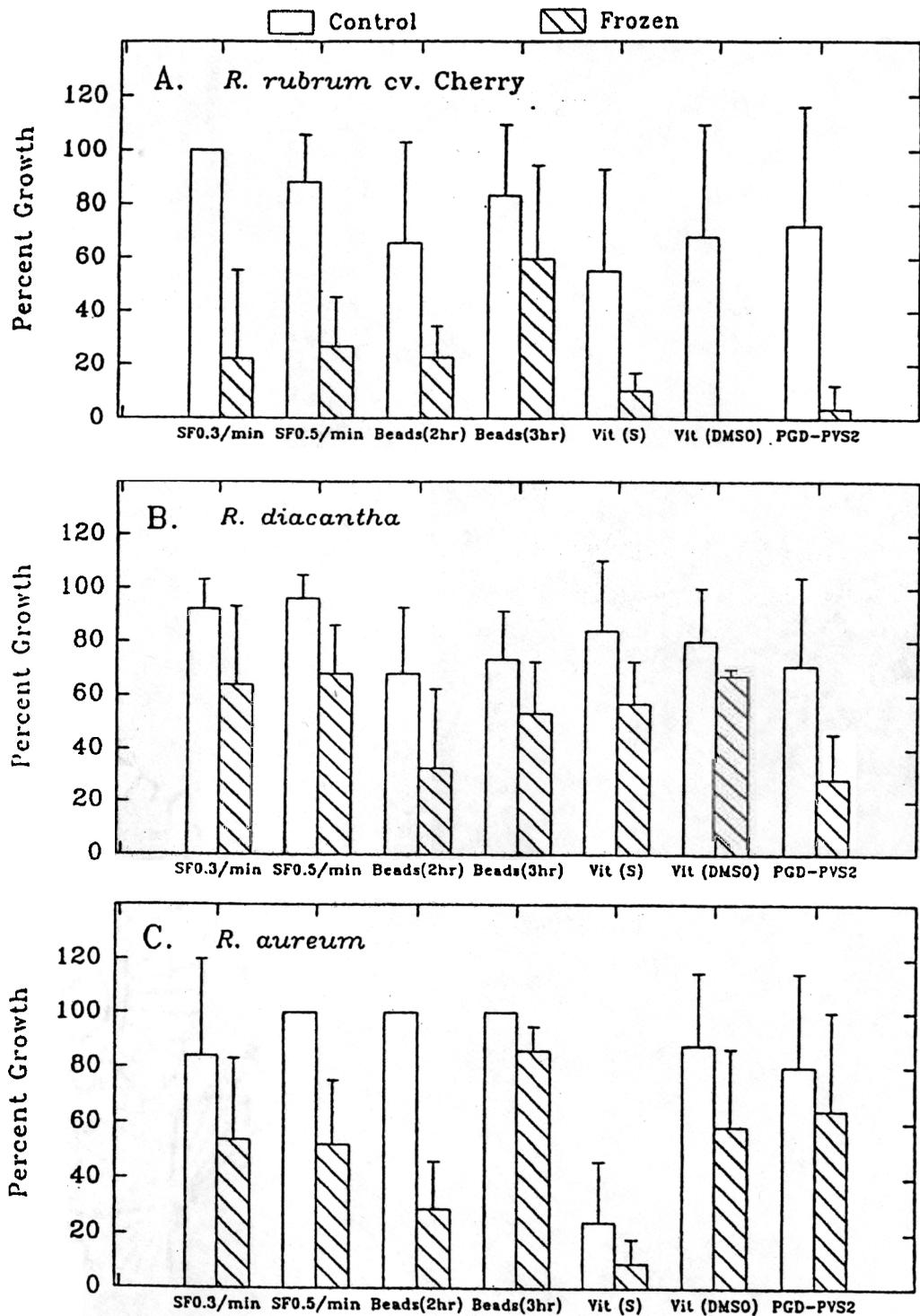


Figure 1. Survival of control and frozen meristems of three *Ribes* genotypes following cryoprotection or cryoprotection and freezing with 7 different techniques (Mean \pm standard deviation). Slow Freezing (SF) at 0.3 or 0.5 $^{\circ}$ /min; Encapsulation-dehydration with 2-hr or 3-hr dehydration (Beads); Vitrification with sorbitol pretreatment (Vit (S)); or DMSO pretreatment (Vit (DMSO)); Vitrification with a PGD pretreatment (PGD-PVS2) A. *Ribes rubrum* cv. Cherry. B. *Ribes diacantha*. C. *Ribes aureum*.

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