

Medium and Long-Term Storage of *Rubus* Germplasm

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Abstract

The United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository at Corvallis, Oregon preserves genetic resources for *Rubus*. The in vitro collection includes about 200 accessions. In vitro cold storage of these accessions is at 4°C with 12 h of low light. Storage facilities for germplasm collections vary, but one week of cold acclimation followed by 4°C storage in the dark or with a photoperiod is acceptable for most *Rubus* germplasm when quarterly evaluation inventories are used to determine timing of repropagation. A reduced-nitrogen medium extends room temperature storage to nine months and is a useful alternative for cold sensitive and tropical genotypes which typically only survive for a short time in cold storage. Meristems of 34 cold-acclimated genotypes of *Rubus* (blackberry and raspberry) were successfully cryopreserved by slow cooling through optimization of cryoprotectants, cooling rates and cold acclimation. Alternating low temperatures as a cold acclimation (CA) treatment improved recovery of shoot tips cryopreserved by slow freezing. The length of the CA required varied from 1 to 10 weeks and was genotype dependent. Cryopreserved *Rubus* shoot tips produced shoots directly from either apical meristems or axillary buds, but not from callus. Shooting increased and callus formation decreased when IBA was eliminated from the recovery medium. Shoot tips of 25 genotypes in 9 *Rubus* species were successfully cryopreserved using encapsulation-dehydration with recovery of 60–100%. Four genotypes of 3 species were tested using PVS2 vitrification with 71% average regrowth. A protocol for cryopreservation of *Rubus* germplasm should include a CA period of 2–10 weeks and recovery on auxin-free medium. These studies confirm that all three cryopreservation protocols may be used for cryopreservation of a wide range of *Rubus* genetic resources.

INTRODUCTION

The United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository (NCGR) at Corvallis, Oregon, preserves genetic resources for *Rubus* (Hummer, 1989; Hummer, 1996). More than 1500 *Rubus* clones and seeds representing more than 150 species are maintained in this genebank. The main collections are potted plants in screenhouses with in vitro culture as a secondary backup and cryopreservation in progress for some accessions. The in vitro collection includes about 200 accessions. Clonally propagated *Rubus* accessions require constant protected pot culture due to their general invasive character in field plantings and the spread of virus diseases by insect vectors. In vitro culture techniques including slow growth storage and cryopreservation provide alternative storage forms for protecting important germplasm (Ashmore, 1997; Reed et al., 2005). Research on the medium and long-term storage of *Rubus* cultures is ongoing at NCGR. This paper summarizes the techniques used for in vitro and cryopreserved storage of

MATERIALS AND METHODS

In Vitro Storage

In vitro-grown *Rubus* sp. plantlets were micropropagated in Magenta GA7 boxes (Magenta Corp., Chicago, IL, USA) on NCGR-RUB medium, a modified MS medium (Murashige and Skoog, 1962) with doubled EDTA-Fe, 4.4 μM N⁶-benzyladenine (BA), 0.49 μM indole-3-butyric acid (IBA), 0.35% agar (Bitek agar, Difco, Detroit, MI, USA), 0.145% (w/v) Gelrite (Kelco, San Diego, CA), and 3% (w/v) sucrose. The pH was adjusted to 5.8 before autoclaving. The plantlets were subcultured every three weeks, and grown at 25°C with a 16 h light/8 h dark photoperiod ($40 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) (Reed, 1990).

1. Cold Acclimation. Plantlets (2–3 cm) were planted in sealed tissue culture bags and placed in the growth room for 1 week, then they were cold acclimatized (CA) with alternating temperatures and a short photoperiod and low radiance [22°C with 8 h light ($10 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)/-1°C 16 h dark] for 1 week prior to cold storage.

2. Cold Storage. Ten plantlets of each accession were stored, each in an individual section (15 x 150 mm) of a five-section bag (Gardner Enterprises, Willis, TX) with 10 ml of medium per section. Bags contained a firmer medium (3.5 g agar and 1.75 g Gelrite per liter) compared to that used in boxes to compensate for the small amount of water lost through the semi-permeable bag walls and no growth regulators (Reed, 1993a). Every four months the bags of plants were inventoried and each bag rated as a whole on a scale of 1 to 5, with a rating of: 5 = all in excellent condition (green shoots, leaves present), 4 = good (elongated shoots, shoot tips healthy), 3 = fair (some browning, some shoot tip necrosis), 2 = poor (extensive browning, most shoot tips necrotic), and 1 = very poor (viability questionable, brown, shoots necrotic) (Reed, 1992).

3. Reduced Nitrogen Storage at Room Temperature. Four nitrogen concentrations (0%, 6%, 25%, 100% MS nitrogen) were varied for the basal medium (10 or 20 ml per section). Plantlet storage on the 4 concentrations was compared for 25°C growth-room storage of *Rubus* cultures. A control set of cultures on the standard medium (100% N) was also stored at 4°C (Reed, 1993a).

Cryopreservation

In vitro-grown cultures were used as a source of shoot tips for cryopreservation. Plantlets were grown as noted above and three weeks after the last subculture were cold acclimated for 1 to 6 weeks. Plantlets in magenta boxes were cold acclimated for 1–10 weeks without transfer. Plantlets were placed in cold acclimation (CA) 3 weeks after subculture. Conditions were the same as for cold storage. Shoot tips (0.8–1.0 mm) were taken from the cold acclimated plantlets and pretreated as noted (Chang and Reed, 1999).

1. Slow Cooling. Dissected shoot tips from CA treatments and control treatments (0.8–1.0 mm) were grown for 48 h on NCGR-RUB medium with 5% dimethyl sulfoxide (DMSO) under the same CA conditions as the parent plantlets. Following the 48 h 5% DMSO pretreatment, shoot tips were transferred to 1.2 ml plastic cryo-vials containing 0.25 ml liquid NCGR-RUB medium at 0°C. The cryoprotectant PGD (Ulrich et al., 1979) [a mixture of 10% each polyethylene glycol (MW 8000), glucose, and DMSO in liquid NCGR-RUB medium] was added up to 1.2 ml over 30 min (Chang and Reed, 1999; Reed and Lagerstedt, 1987). After 30 min equilibration at 0°C, the shoot tips were cooled to -35°C at 0.5°C/min in a programmable freezer (CRYO-MED, Forma Scientific, Mt. Clemens, Mich.) and then immersed in LN for 1 h. Vials were thawed in 45°C water for 1 min, then for 2 min in 23°C water. The cryoprotectant was removed and replaced with liquid NCGR-RUB medium. Shoot tips were recovered on NCGR-RUB medium without IBA in 24 cell plates, one shoot tip per cell (Costar, Cambridge, Mass.).

2. Histology. *R. parvifolius* meristems recovered on NCGR-RUB medium without IBA were fixed in FAA (50% alcohol: formalin: acetic acid =18:1:1) at 0, 3, 5, 7, 11, 15 and 21 days following thawing. After at least 48 h fixation, the samples were dehydrated with

tert-butyl-alcohol (TBA) and water series, infiltrated with xylene and embedded in paraffin. The sections (10 µm thick) were cut with a rotary microtome and stained with 1% safranin and 0.1% fast green.

3. PVS2 Vitrification. The PVS2-vitrification protocol for white clover was modified for this study (Yamada et al., 1991). Excised shoot tips were pretreated for 48 h on MS medium containing 5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St. Louis, MO)] (v/v) with 3.5 g agar and 1.75 g/L gelrite under the standard cold-acclimation conditions. Shoot tips were transferred into 1.2 ml cryo-vials and treated with 1 ml loading solution (LS) (2M glycerol in 0.4 M sucrose solution) (Sakai et al., 1991) for 20 min at either 0°C or 25°C. LS was removed and PVS2 cryoprotectant solution [(v/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in liquid MS medium with 0.4 M sucrose at pH 5.8] was added into cryovials for 15, 20, 30 or 40 min at either 0°C or 25°C. After PVS2 treatment, vials were submerged in LN for 1 h. Rewarming was as described above. Shoot tips were immediately rinsed twice in liquid MS medium containing 1.2 M sucrose and planted on recovery medium as described above.

4. Encapsulation Dehydration. The method developed for pear was used with some modifications (Dereuddre et al., 1990). Excised shoot tips were encapsulated in alginate beads composed of 3% (w/v) low viscosity alginic acid (Sigma Chemical Co. USA) in liquid MS medium without calcium, pH 5.7 and allowed to polymerize for 20 min in MS medium with 100 mM CaCl₂ and 0.4 M sucrose. Beads were pretreated in liquid MS medium with 0.75 M sucrose for 20 h on a rotary shaker (50 rpm), blotted dry on sterile filter paper and dehydrated for 6 h [~20% moisture content] in a glass Petri dish under laminar flow at ambient temperature (~25°C). Dried beads were placed in 1.2 ml cryovials (10 beads/cryovial) and plunged directly into liquid nitrogen (LN). The vials were reheated in 45°C water for 1 min and 25°C water for 2 min. The beads were rehydrated in liquid MS medium for 5 min and transferred onto recovery medium (RUB with no IBA) in 24 cell plates for regrowth (Costar, Cambridge, Mass.) (Chang and Reed, 1999). Three sets of 20 dehydrated beads were used to determine bead moisture content. Three replicates of 25 beads (n=75) were weighed and dried at 103±2°C for 17±1 h. Total water content expressed as % FW and was determined as the difference between dry weight (DW) and fresh weight (FW) calculated as ((FW-DW)/FW) x 100. Grams of water per gram dry weight was also determined (FW-DW/DW).

RESULTS AND DISCUSSION

In Vitro Storage

1. Cold Storage. Initial in vitro-storage studies of *Rubus* hybrids 'Malling Promise' and 'Kotata', *R. leucodermis* Dougl. and *R. caesius* L. genotypes showed that shoot cultures stored in tissue culture bags were in significantly better condition than plantlets stored in 20 x 150 mm tubes. Plantlets stored with a 12 h photoperiod at 4°C also stored longer than those held in the dark. Cultures stored at 10°C survived only 6 months. Evaluation of 250 accessions after 12 months of storage at 4°C in the dark showed that 92% of accessions in bags and 85% of those in tubes remained in good condition for continued storage. Genotype variation was very high, as would be expected for a widely diverse germplasm collection (Reed, 1993a). As a result of these studies *Rubus* shoot cultures at NCGR are stored in plastic tissue culture bags at 4°C with 12 h of low light (Fig. 1) on a modified MS medium with no growth regulators (Reed and Chang, 1997).

2. Reduced Nitrogen Storage at Room Temperature. Cold storage is often detrimental to accessions with tropical or subtropical origins. Six to 9 month subculture periods were possible for shoot cultures stored at 25°C in tissue culture bags with reduced nitrogen. The volume of medium was critical to a longer subculture period as was the N in the medium. Plantlets grown on 10 ml medium could be stored for only 6 months due to dehydration of the medium. Survival of 'Mandarin' raspberry stored for 9 months on 20 ml of medium with 25% of the normal MS nitrogen was 90% compared to 40% for shoots grown on 6% or 100% N or at 4°C with 100% N (Reed, 1993a). These results are similar

to those of Moriguchi and Yamaki (1989) with chilling sensitive *Vitis* cultures.

Cryopreservation

1. Slow Cooling. Initial studies of *Rubus* shoot tip cryopreservation showed that slowly cooling shoot tips in the cryoprotectant solution PGD was effective for *R. spectabilis* Pursh and *Rubus* species 'Merton Thornless' (Reed and Lagerstedt, 1987). The addition of one week of CA significantly improved the regrowth of shoot tips for two other accessions, *R. idaeus* L. 'Heritage' and *Rubus* species 'Logan Thornless' (Reed, 1988). ABA was shown to improve the effect of CA for *Rubus* hybrid 'Hillemeier' and *R. cissoides* Cunn. by significantly improving regrowth compared to CA alone. Three other genotypes were negatively impacted by the addition of ABA and it could not substitute for CA in any of the five genotypes tested (Reed, 1993b). In all of these studies a high percentage of the recovering shoot tips produced callus, but it did not generate shoots. Removal of the auxin IBA from the recovery medium for *Rubus* shoot tips eliminated or greatly reduced callus production (Chang and Reed, 1999). Increased duration of CA greatly improved the regrowth of cryopreserved *Rubus* meristems that gave low or no survival after a 1-week CA period. When the cold period was short, survival was low and most of the surviving shoot tips formed callus rather than shoots. As the duration of CA prior to cryopreservation increased from 1 to 3 weeks, *R. parvifolius* L. shoot regrowth increased from 25% to 75%. More than six weeks of CA was required to achieve high shoot regrowth in *R. caesius* L. This phenomenon indicates that the apices were seriously injured during the freezing/thawing process of cryopreservation, resulting in callus production during regrowth. Tissues and cells which have been cold acclimated for longer periods were more resistant to freezing stress and therefore were better able to recover following cryopreservation (Chang and Reed, 1999). No shoots regenerated from callus produced on margins of leaf primordia and damaged meristematic domes (Chang and Reed, 1999; Reed, 1993b). Histological studies showed that *R. parvifolius* shoot tips grew directly from meristematic domes following liquid nitrogen exposure. Axillary buds often survived and regrew along with the apex. Meristems of 34 cold-acclimated genotypes of *Rubus* (blackberry and raspberry) were successfully cryopreserved by slow cooling through optimization of cryoprotectants, cooling rates and cold acclimation (unpublished data).

2. PVS2-Vitrification. Four genotypes of 3 species were tested using the vitrification protocol with 71% average regrowth. The optimization of pretreatments, PVS2 exposure and regrowth protocols all contribute to the recovery of shoots following vitrification procedures. In this case 20 minutes in PVS2 at 25°C produced the best regrowth. 'Burbank Thornless', 'Jingu Juegal', and 'Chehalem' had significantly better shoot growth (65–78%) than 'Hull Thornless' (45%) (Fig. 2). All shoot tips grew into shoots without undergoing a callus phase (Gupta and Reed, 2006).

3. Encapsulation-Dehydration. Recovery of air-dehydrated apices as well as LN treated apices, improved significantly ($P \leq 0.05$) with increasing CA. The maximum shoot regrowth after LN (85%) was reached after 4 weeks of CA. The encapsulation and osmotic dehydration steps did not affect viability, but up to 20% viability was lost following air dehydration. The viability of some genotypes was further reduced by LN exposure. Regrowth following LN ranged from 60% to 100%. The protocol worked successfully for both blackberry and raspberry cultivars (Fig. 2). Plants did not produce callus at any stage of recovery. Shoot tips of 25 genotypes in 9 *Rubus* species and 9 *Rubus* hybrids were successfully cryopreserved with recovery of 60–100% using the encapsulation-dehydration protocol (Gupta and Reed, 2006). A recent study found that a slightly different encapsulation-dehydration technique tested on 7 *Rubus idaeus* selections produced a mean 55% regrowth while an encapsulation-vitrification technique produced a mean 75% regrowth for 7 genotypes and 68% for 4 others (Wang et al., 2005).

CONCLUSIONS

Storage of germplasm collections could incorporate whichever technique is the most convenient. The amount of technical assistance available and the number of

accessions to be stored may determine which technique to use. Many factors are involved in this decision and these should be evaluated before storage is begun (Reed, 2002; Reed et al., 2005). Cold acclimation is an effective pretreatment for *Rubus* shoot tips for both in vitro cold storage and cryopreservation by any of the major techniques. Cold-tolerant *Rubus* shoots stored in vitro at 4°C can be held for an average of 2.2 years. Cold-sensitive shoots stored at growth-room temperatures can be held for 9 months without transfer. *Rubus* cultivars regrown after cryopreservation with slow cooling, PVS2 vitrification, and encapsulation dehydration had good recovery. A wide range of *Rubus* germplasm was cryopreserved by slow cooling, vitrification and encapsulation dehydration. All three cryopreservation techniques produced good recovery and growth of cryopreserved *Rubus* shoot tips into plantlets.

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Figures



Fig. 1. Plantlets of in vitro-grown *Rubus* accession ‘Aurora’ after 9 months of cold storage at NCGR, Corvallis, OR.

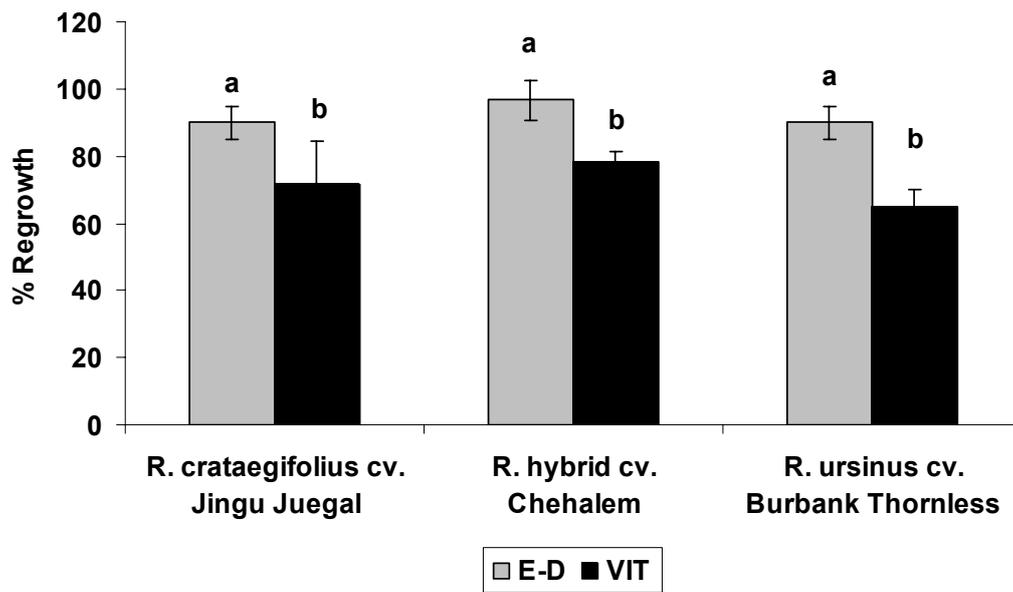


Fig. 2. Regrowth of shoot apices of *Rubus* spp. cryopreserved using encapsulation-dehydration and vitrification protocols. Data are means \pm SD. Means for a genotype followed by different letters are significantly different at $P \leq 0.05$.

