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Cryopreservation of Raspberry Cultivars: Testing Techniques for Long-Term Storage of Kazakhstan's Plant Germplasm

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ABSTRACT

The genetic resources of vegetatively propagated plants are usually maintained in field genebanks. *In vitro* methods play an important role as backup collections for these irreplaceable plants. Medium-term backups can be held as *in vitro* cultures in cold storage for several years, but also remain at risk of loss. In order to provide long-term storage of raspberry genetic resources, i*n vitro* cultures of raspberry cultivars and selections held in the Kazakhstan national germplasm collection were tested for cryopreservation in liquid nitrogen. Three techniques were tested, two with PVS2 vitrification and one with encapsulation dehydration. Cold acclimation (CA) can be used to improve regrowth following cryopreservation, so the optimum CA duration was determined for two cultivars. Three weeks of CA was optimal for the best regrowth of raspberry 'Druzhnaya' while 3 to 5 weeks were best for 'Anar'. Three months of CA did not improve regrowth for 'Anar'. Both cultivars had good regrowth following the 0.3M sucrose pretreatment and the PVS2 vitrification technique. 'Anar' responded equally well to the pretreatment with 5% DMSO and 1% bovine serum albumen followed by PVS2 vitrification. Cryopreservation with the encapsulation dehydration technique produced significantly less regrowth in both cultivars. Cultivars and selections Anar, Babiye Leto, Druzhnaya, Kerzhach, Kokinskaya, K-10-12, K-12-4, K-13-60, Latham, Osnovyanka, and Pathfinder were cryopreserved with the sucrose pretreatment and PVS2 vitrification technique and stored in liquid nitrogen dewars. Initial results after short liquid nitrogen exposure showed 52-82% regrowth from nine of the eleven raspberries. Samples remaining in liquid nitrogen for long-term storage will be assessed later. This study indicates that cryopreservation is a reasonable technique for long-term storage of raspberry germplasm.

Keywords: encapsulation dehydration, genetic resources, liquid nitrogen, PVS2, *Rubus*, vitrification
Abbreviations: CA, cold acclimation; **DMSO**, dimethylsulfoxide; **MS medium**, Murashige and Skoog (1962) medium; **PVS2**, Plant vitrification solution number 2; **Vit (M&S)**, Matsumoto and Sakai (2000) medium; **Vit (L&R)**, Luo and Reed (1997) medium

INTRODUCTION

Genetic resources of fruit and berry crops of Kazakhstan are very important both for their scientific and practical value. Kazakhstan holds a plant germplasm collection with a wide diversity of wild forms and unique selections from the wild, adapted local and foreign cultivars, old cultivars of local breeders and unique hybrid materials. Genetic resources are the national property of every country and care must be taken to maintain and protect them.

Breeding of new cultivars and improvement of old ones requires a range of genetic materials. Often the genetic resources available are insufficient for meeting breeding goals. Genetic diversity in germplasm collections provides the opportunity to breed new cultivars resistant to insects, diseases and stress and this diversity can be introduced from wild species or closely-related species of cultivated plants. As the natural areas that maintain these wild species continue to be reduced, many species disappear forever; to conserve this genetic diversity it is important to preserve them, both *ex situ* and *in situ* (Lynch *et al.* 2007).

One strategy for preserving *ex-situ* genetic diversity is the creation of a germplasm bank using *in vitro* biotechnological methods (Engelmann 1997; Benson 1999). The main biotechnological methods of germplasm preservation for vegetatively propagated fruit and berry plants are the micropropagation of shoots, cold storage of the cultures (4°C) and cryopreservation (-196°C). *In vitro* and field germplasm collections are considered to be complementary. Conservation and preservation of fruit genetic resources in Kazakhstan includes three forms of storage: preservation of field collections for evaluation and immediate use by breeders, cold storage at low temperature (4°C) for medium-term storage, and cryopreservation of shoot tips in liquid nitrogen (-196°C) for long-term storage.

In the last 20 years techniques for cold storage and cryopreservation were developed and successfully applied to many types of plants (Reed 2008). Several standard techniques, PVS2 vitrification (Sakai *et al.* 1990; Sakai 2004; Sakai *et al.* 2008), encapsulation dehydration (Engelmann *et al.* 2008) and others (Reed 2008) are now available for use. Cryopreservation of some *Rubus* genotypes including cultivars and wild species was developed using controlled rate cooling (Reed and Lagerstedt 1987; Reed 1993; Chang and Reed 1999) and many genotypes were stored. Raspberry cryopreservation using PVS2 vitrification, encapsulation dehydration and encapsulation vitrification was tested for some genotypes by Vysotskaya *et al.* (1999), Wang *et al.* (2005), Gupta and Reed (2006) and Reed *et al.* (2008). These standard techniques are now available for use with many plants; however they may require optimization under varying laboratory conditions.

This study was designed to test the efficacy of three cryopreservation techniques for the long-term storage of unique raspberry germplasm held in Kazakhstan and to begin a long-term storage collection of *Rubus* germplasm.

MATERIALS AND METHODS

All chemicals were purchased from PhytoTechnology Laboratories (Shawnee Mission, KS, USA), were reagent grade and tissue culture tested.

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Plant materials and growth conditions

Raspberry cultivars and selections Anar, Druzhnaya, Kokinskaya, K-10-12, Babiye Leto, Pathfinder, K-12-4, Latham, Osnovyanka, Kerzhach, and K-13-60 from the *in vitro* collection of the Pomological Garden of the Research Institute of Horticulture and Viticulture in Almaty were used in these experiments. Raspberry plantlets were micropropagated on MS medium (Murashige and Skoog 1962) with doubled EDTA-Fe, 1 mg/l benzyl amino purine (BAP), 0.1 mg/l indole-3-butryic acid (IBA), 3.5 g/l agar, 1.75 g/l Gelrite, and 30 g/l sucrose at pH 5.8. The plantlets were grown at 23-25°C with a 16-h light (25 $\mu mol \cdot m^{-2} \cdot s^{-1}$) photoperiod. Cultures were transferred to fresh medium at three week intervals. Recovery medium used after cryopreservation was the medium above without IBA and with 3.0 g/l agar, 1.3 g/l Gelrite (Reed 1990).

Cold acclimation (CA)

Shoot cultures were CA in a growth chamber (Lab-Line Environette, Melrose Park, IL, USA) with alternating temperatures [22°C with 8 h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) / -1°C 16 h darkness]. *In vitro* plantlets were acclimated for 0 to 6 weeks. Cultivars Anar and Druzhnaya were tested with the modified Matsumoto and Sakai (2000) technique (below) to determine the optimal amount of cold acclimation (0 to 6 weeks). 'Anar' was also evaluated after 3 months CA.

Cryopreservation procedures

Cultivars Anar and Druzhnaya were used to compare three cryopreservation procedures. Plantlets for all three techniques were cold acclimated for 3 weeks, as described above, prior to use in these experiments. Shoot tips (0.8 to 1.0 mm) were dissected from CA *in vitro*-grown shoots.

1. PVS2 vitrification with sucrose preculture

PVS2 vitrification with a modified sucrose preculture was tested (Matsumoto and Sakai 2000). Shoot tips were dissected from 3-week CA plants and precultured for 2 days in CA conditions on 0.3 M sucrose MS agar medium; the original method did not use cold acclimation and pretreatment was for 3 days at 25°C. After preculture, shoot tips were placed in loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) for 20 min and then transferred to 1.2 ml cryovials on ice with 1 ml PVS2 (30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) in 0.4 M sucrose liquid MS medium at pH 5.8) for 80 min. Vials were then submerged in liquid nitrogen. For rewarming vials were removed from the liquid nitrogen and immediately plunged into a 45°C water bath for 1 min, then transferred to 22°C water for 1 min. Shoot tips were rinsed in liquid 1.2 M sucrose MS medium twice for 1-2 min each time, drained on filter paper and placed on the recovery medium (listed above) for regrowth. Controls (5 shoot tips per treatment) were pretreated, exposed to loading solution and PVS2 solution, then rinsed with liquid medium and plated on recovery medium.

2. PVS2 vitrification with 5% DMSO preculture

This technique was developed for *Ribes* shoot tips (Luo and Reed 1997). Shoot tips were dissected from 3-week CA plants and precultured for 2 days in CA conditions on MS agar medium with 5% DMSO. Then shoot tips were placed in MS medium with 1% (w/v) bovine serum albumin (BSA) for 2 h. BSA was removed and 1 ml PVS2 was added to the cryotubes on ice for 20 min and the tubes were submerged in liquid nitrogen. Rewarming of samples was carried out as above. Controls (5 shoot tips per treatment) were pretreated, exposed to BSA and PVS2 solutions, then rinsed with liquid medium and plated on recovery medium.

3. Encapsulation dehydration

The method developed by Dereuddre (1990) and modified by Reed (2001) was used. Shoot tips dissected from 3-week CA plants were placed in low viscosity alginate [3% alginate in 0.75 M sucrose liquid MS medium without calcium at pH 5.7] and dispensed from a pipette into a saturated calcium chloride solution to make beads. Beads were held in the solution for 20 min to polymerize, then transferred to flasks of 0.75 M sucrose liquid MS medium on a shaker for 18 h. After 18 h the beads were drained, blotted dry and placed in sterile Petri dishes in the air flow of a laminar hood for 4 h (20% bead moisture content), placed in cryovials and submerged in liquid nitrogen. Rewarming of cryovials was carried out at room temperature for 20 min, then the shoot tips in alginate beads were rehydrated in liquid MS medium for 5 min and placed on recovery medium. Controls (5 shoot tips per treatment) were encapsulated, treated in sucrose, dried and rehydrated before plating.

Germplasm storage

Eleven additional *Rubus* genotypes were cryopreserved with the modified Matsumoto and Sakai (2000) vitrification technique. Two or three tubes of each genotype, 20 shoot tips/tube, were stored. One tube was removed, rewarmed and regrown as a control to test viability after 24 h. This study was not replicated. The remaining tubes will remain in long-term storage in liquid nitrogen until needed. Controls (5 shoot tips/treatment) were treated as for Matsumoto and Sakai (2000) vitrification technique above.

Data and statistical analysis

Each experiment was done three times and consisted of 20 shoot tips per treatment (n=60). Controls for each experiment consisted of 5 treated shoot tips per treatment, exposed to all solutions, but not exposed to liquid nitrogen, rinsed and plated with the experimental treatments. Regrowth data was taken at 6 weeks. Data is presented as the mean of the replicates ± standard deviation. Regrowth means were compared with a 1-tailed *t*-test (Systat 2007).

RESULTS AND DISCUSSION

Cold acclimation

The effectiveness of CA was tested with two cultivars cryopreserved with the modified sucrose preculture (Matsumoto and Sakai 2000). Regrowth of 'Druzhnaya' cryopreserved shoot tips increased up to 4 weeks and then declined, with significantly better recovery ($P \leq 0.05$) at 3 and 4 weeks than the other treatments (**Fig. 1**). 'Anar' regrowth increased greatly with 1 week CA, then recovery remained at moderate levels through the remaining weeks with no significant difference ($P \leq 0.05$) among the 1 through 6 week CA treatments. Regrowth of 'Anar' did not significantly improve (55% regrowth) after 3 months CA (data not shown). All further experiments were done with 3 week CA. Regrowth of controls (no liquid nitrogen exposure) was always 80-100% (data not shown). Cold acclimation was found to be very important for other *Rubus* genotypes where three to as long as 10 weeks of alternating CA were required for high recovery following cryopreservation (Chang and Reed 1999). This is similar to what is seen with the cold acclimation of pear shoots where there was a range of variability among cultivars (Chang and Reed 2000). In that case, extended cold acclimation sometimes improved recovery from cryopreservation, but the time required was unique to each genotype tested and depended on the amount of cold hardiness acquired (Chang and Reed 2001). Alternating temperature acclimation was also shown to be more effective for mint shoot tip cryopreservation than a static low temperature (Senula *et al.* 2007). Palonen and Junttila (1999) demonstrated that adding additional sucrose to the medium during a 14 day CA gave even better cold hardiness to raspberry plants than cold temperatures alone. This may be a useful tactic to use with genotypes that do not respond well to standard CA.

Techniques

One of the quickest ways to determine and effective cryo-

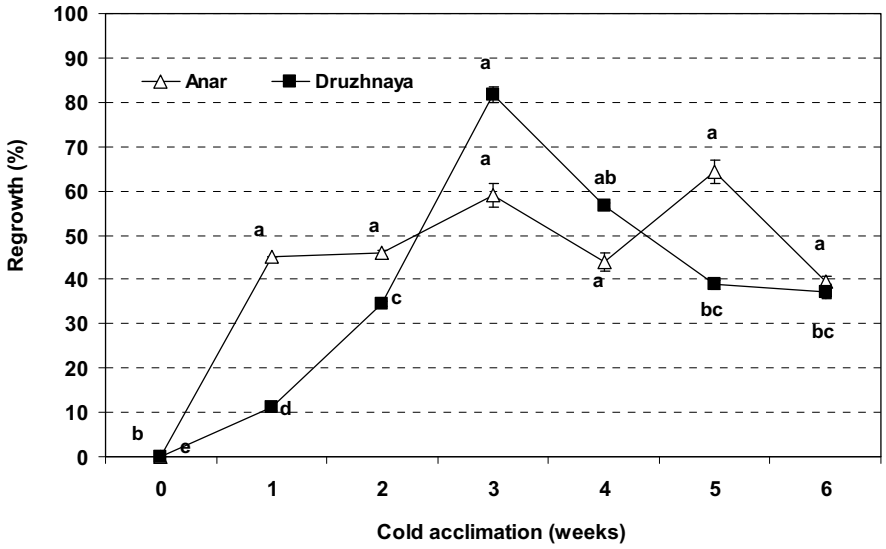

Fig. 1 Recovery at 6 weeks after rewarming (mean ± standard deviation) of two Kazakh raspberry cultivars following cryopreservation by PVS2 vitrification by the method of Matsumoto and Sakai (2000) after 0 to 6 weeks of alternating-temperature cold acclimation. Means for a cultivar followed by the same letter are not significantly different as compared by t-tests. n=60.

preservation technique is to test several standard techniques with a range of germplasm (Reed 2008). In this case two variations of PVS2 vitrification and the encapsulation-dehydration technique were tested. Regrowth for 'Druzhnaya' following liquid nitrogen exposure was significantly better ($P \leq 0.05$) with the modified Matsumoto and Sakai technique (2000) using cold acclimation, a 0.3 M sucrose pretreatment and 80 min exposure to PVS2 than the other techniques (**Fig. 2**). 'Anar' regrowth was not significantly different ($P \leq 0.05$) for the two vitrification techniques, and both were significantly better than encapsulation dehydration. Both cultivars reached the 40% minimum regrowth required for safe storage (Reed 2001; Dussert *et al.* 2003). The poor response of these cultivars to encapsulation dehydration (E-D) contrasted with the successful storage (60-100% regrowth) of 25 *Rubus* genotypes in 9 species with the standard E-D technique (Reed *et al.* 2008). An E-D procedure that included 0.4 M sucrose and 2M glycerol in 2.5% alginate beads was very effective for seven *Rubus idaeus* selections (average of 55% regrowth) (Wang *et al.* 2005). Sometimes optimization of a technique is needed for the best regrowth after cryopreservation. Our cryopreservation tests did not track the survival of the shoot tips during the procedure and this might be needed to indicate which part of the procedure was lethal for the plants. Increased sucrose loading or increased dehydration may improve recovery for these genotypes. Fast rewarming of the beads in warm water might also improve regrowth as was seen in other species. Drying the beads over silica gel would be a possible option for standardizing the drying protocol. Various changes in the PVS2 vitrification technique can affect regrowth as well. PVS2 vitrification was successful for four *Rubus* genotypes (average of 71% regrowth) following a 4-week CA, a 5% DMSO pretreatment and 20 min in a glycerol and sucrose loading solution (Gupta and Reed 2006). These results are similar to the 40-82% regrowth from the two Kazakh cultivars (**Fig. 2**).

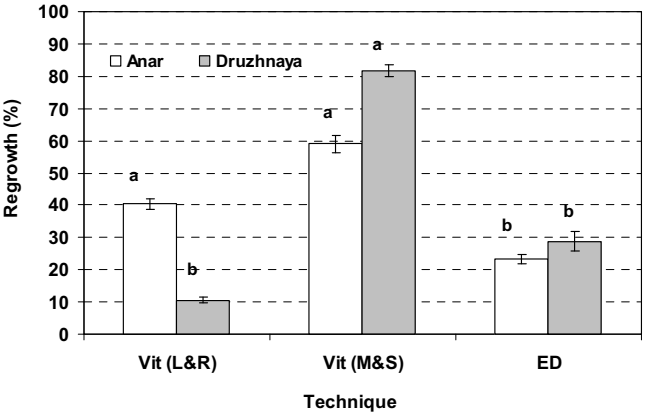

Fig. 2 A comparison of three cryopreservation techniques with two Kazakh raspberry cultivars, Anar and Druzhnaya. Regrowth is after 6 weeks on recovery medium (mean ± standard deviation). Both cultivars were cold acclimated for 3 weeks, then shoot tips were cryopreserved using the vitrification techniques of Luo and Reed (1997) Vit (L&R), or Matsumoto and Sakai (2000) Vit (M&S), or encapsulation dehydration (ED). Means for a cultivar followed by the same letter are not significantly different as compared by *t*-tests. n=60.

Long-term storage of raspberry germplasm

Cryopreservation is intended to be a solution to the problem of long-term storage for vegetatively propagated germplasm (Benson 1999; Engelmann 2004). Most cryopreservation techniques are developed with one or two genotypes but are not applied to the genetic diversity available in national collections. It is not possible to develop a new technique for every accession in a large collection, however screening of germplasm with well tested techniques can provide indications of promising techniques (Reed *et al.* 2003). In this study, 11 raspberry genotypes were screened and stored for long-term preservation of the germplasm (**Fig. 3**). This was a single storage event and was not replicated. All the cultivars and selections were cryopreserved with 3 weeks of CA, 2 days of 0.3 M sucrose pretreatment in CA conditions and 80 min in PVS2. After liquid nitrogen exposure, regrowth after 6 weeks ranged from 20 to 81% (**Fig. 3**). Only two cultivars had regrowth that was below the 40% desired for safe germplasm storage (Reed 2001). Additional vials of these low performing genotypes could be added to provide enough viable shoot tips for recovery of the germplasm (Matsumoto and Sakai 2000; Dussert *et al.* 2003). Additional vials of each genotype will remain in liquid nitrogen as the beginning of the cryostored collection.

CONCLUSIONS

Cryopreservation of the raspberry germplasm of Kazakhstan is now a reality. Storage of eleven genotypes in liquid nitrogen is the beginning of a long-term storage collection. While there is some variability in the response of the cultivars, it is clear that most can be stored with one technique. Those with low viability may require increased cold

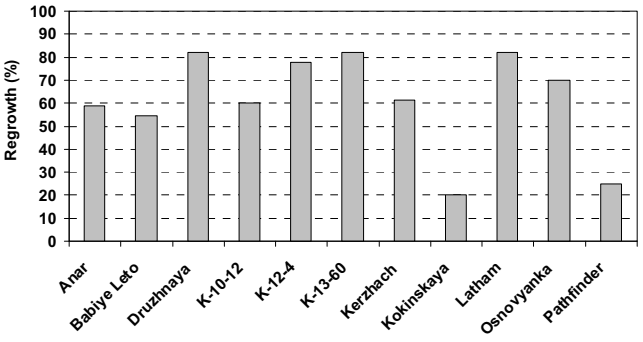

Fig. 3 Regrowth of eleven raspberry cultivars and selections 6 weeks after rewarming following cryopreservation in liquid nitrogen for 24 hours. Each bar represents one vial of 20 shoot tips to indicate the initial viability after liquid nitrogen exposure. Additional vials remain in liquid nitrogen for long-term storage. A modified vitrification technique based on Matsumoto and Sakai (2000) was used.

acclimation, improved growth medium or the use of another technique. This study indicates that raspberry genetic resources of Kazakhstan can now be safely stored in liquid nitrogen as a long-term backup to the active field and *in vitro* collections maintained at the Pomological garden of Research Institute of Horticulture and Viticulture in Almaty.

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