Biodiversity and Conservation **10**: 939–949, 2001.
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Validation of cryopreservation protocols for plant germplasm conservation: a pilot study using *Ribes* L.*

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Received 10 March 2000; accepted in revised form 16 July 2000

Abstract. Uniformly applicable techniques for germplasm preservation are important to the international genetic resources community and validation of techniques among working genebanks will enable the integration of new technologies into plant genetic resources programs. Apical meristems from micropropagated plants of *Ribes nigrum* L. cv. Ojebyn and *R. aureum* cv. Red Lake were used to test three cryopreservation protocols (controlled freezing, plant vitrification solution no. 2 (PVS2) vitrification and encapsulation–dehydration) at the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, OR, USA and the University of Abertay Dundee (UAD), Scotland. Similar results were obtained with PVS2 vitrification at both locations but meristem regrowth varied greatly for the other techniques. Variable results between the locations were noted for controlled freezing and were largely attributed to differences in ice crystal initiation by the controlled rate freezers. Low survival of 'Red Lake' at UAD with all three techniques was attributed to poorly performing shoot cultures. Attention to protocol details is important for limiting variation between locations and step by step instructions for procedures and solution preparation aided protocol standardization. These studies suggest that source plant status, cryogenic facilities, and culture conditions may be the most likely causes of variation when validating cryopreservation methodologies in different locations. However, in-house optimization of standard procedures offers considerable potential in ensuring that cryopreservation methodologies can be transferred between international laboratories.

Key words: controlled freezing, cryopreservation, currants, encapsulation–dehydration, germplasm, gooseberry, liquid nitrogen, vitrification

Abbreviations: DMSO – Dimethyl sulfoxide; LN – liquid nitrogen; NCGR – National Clonal Germplasm Repository; PGD – Cryoprotectant solution used in controlled freezing; PVS2 – Plant vitrification solution no. 2; RIB – *Ribes* culture medium; UAD – University of Abertay-Dundee

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Introduction

Preservation of the world's genetic resources is currently at the forefront of conservation activities and biotechnology can play an important role in international plant conservation programs. The effective integration of contemporary technologies, with traditional conservation strategies is important for the successful preservation of plant biodiversity (Callow et al. 1997; Curry and Watson 1998; Benson 1999a). Traditionally, plant genetic resource management involves conserving germplasm as seed at low temperature, or as field plantings (field genebanks) for vegetatively propagated crops. Now these approaches are complemented by *in vitro* conservation methods that can be used in combination with traditional practices and offer added security for field genebank conservation (Ashmore 1997). The ideal genetic resource conservation program consists of active collections that are available for distribution or characterization and base collections held for the sole purpose of long-term preservation. Base collections of seeds are standard, however base collections of vegetatively propagated plants are more difficult to achieve and cryopreservation is now considered the most appropriate option for these systems (Bajaj 1995; Ashmore 1997).

A report from the International Plant Genetic Resources Institute recently highlighted the role of *in vitro* conservation methods in germplasm storage (Ashmore 1997). It will become increasingly important to validate new storage protocols at the international level. *In vitro* conservation comprises two inter-dependent techniques, tissue culture and cryogenic storage. Cryopreservation is the storage of living cells and tissues in liquid nitrogen (LN) at ultra low temperature (−196 °C) and is now applied to a diverse range of plant species and tissue systems (Bajaj 1995; Ashmore 1997; Benson 1999a). The development of many different cryoprotection and cryopreservation methods (Razdan and Cocking 1997; Benson 1999b) has increased the utilization of cryogenic storage for plant germplasm and three main approaches are now available. The first, controlled freezing involves the application of chemical cryoprotectants followed by exposure of plant tissues to a low temperature gradient that is optimized for a critical rate of cooling to a terminal sub-zero transfer temperature. On reaching this point the tissues are transferred to LN. Controlled freezing has many variations (Kartha et al. 1980; Uemura and Sakai 1980; Reed 1988; Towill 1988). The precise control of cooling rates and extracellular ice nucleation is critical to the success of cryopreservation using controlled-freezing methods and such control can only be reliably achieved by using controlled rate, programmable freezers.

Vitrification is the second approach to cryopreservation and involves the pretreatment of germplasm with highly concentrated, chemical cryoprotectant mixtures (Sakai et al. 1991; Niino et al. 1992; Towill and Jarret 1992). In effect vitrification is cryopreservation in the absence of ice, however the glasses formed are highly unstable and great care must be taken to prevent the occurrence of damaging glass relaxation and de-vitrification events upon re-warming. Vitrification solutions can be toxic to

cells, so their application and removal must be precisely controlled in order to avoid cell damage and death.

The third approach to plant germplasm cryopreservation is dehydration (Dereuddre et al. 1990; Fabre and Dereuddre 1990; Plessis et al. 1993). Plant tissues are encapsulated in alginate beads and exposed to osmotic and evaporative dehydration to a critical moisture level. After the dehydration treatment the encapsulated tissue is plunged directly into LN and the water molecules vitrify. Our earlier studies (Benson et al. 1996) characterize the performance of these cryopreservation methods using differential scanning calorimetry. The thermal events associated with chemical vitrification are reproducible for cooling, but care must be taken on re-warming as vitrification solutions become unstable and damaging ice formation may result.

Validation and technology transfer of established and new protocols for use among international genebanks is important for the integration of cryopreservation protocols into traditional plant genetic resource conservation systems. It is important to develop reliable and reproducible methodology that can be applied across a broad genotype range and that can be routinely implemented by different repositories. One of the most important principles of germplasm conservation is that germplasm stocks are duplicated at more than one site (Stacey et al. 1999). It is especially important to ensure that plant cryopreservation methodologies are transferable to operators based in different locations. Good practice procedures are now available for internationally designated microbial and animal culture collections that hold cryopreserved germplasm. However, in the case of plant collections there is little information regarding the implementation of validated procedures at the international level (Stacey et al. 1999).

Techniques for LN storage of clonally propagated plant germplasm are now widely available (Bajaj 1995; Ashmore 1997; Benson 1999a), but are not widely used in genebanks (Reed et al. 1998). For future development of genebank storage it is essential to validate storage protocols in different international laboratories. Such an approach will aid the successful implementation of cryopreservation in plant genebanks worldwide. The objective of this pilot study was to validate three different cryopreservation methods: controlled freezing, vitrification and encapsulation–dehydration in genetic resources laboratories which are based in two locations: the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, OR, USA and the University of Abertay Dundee (UAD), UK.

Materials and methods

General growth conditions and plant materials

Black currant, *Ribes nigrum* L. cv. Ojebyn and red currant, *R. aureum* Pursh cv. Red Lake were micropropagated in both locations using the same protocol. The germplasm was available at NCGR, Corvallis, OR, for the US study and germplasm

donated by the Scottish Crop Research Institute, Dundee, UK for the Scottish study. Micropropagated shoots were multiplied and meristems recovered on NCGR-*Ribes* medium (RIB), which contains the mineral salts and vitamins of Murashige and Skoog (1962) 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 N^6-benzyladenine, 0.2 mg gibberellic acid (GA_3), 6 g agar (Sigma, Poole, Dorset, UK or Bitek, Difco, Detroit, MI, USA), at pH 5.7. Shoots were grown in both locations at 25 °C with a 16-h light (25 μmol m^{-2} s^{-1})/8-h dark photoperiod. All cultures were cold acclimated for 1 week (Reed 1990). At NCGR acclimation was in an incubator with 8-h light at 22 °C and 16-h dark at −1 °C. At UAD acclimation was 8-h light at 25 °C and 16-h dark at 4 °C. After acclimation 0.8 mm apical meristems were excised for cryopreservation and held at the cold acclimating conditions for pretreatment.

Controlled freezing

The method used was developed for *Ribes* (Reed and Yu 1995). Meristems were pretreated for 2 days on RIB medium with 5% dimethyl sulfoxide (DMSO), transferred to 0.25 ml liquid RIB medium in 1.2 ml plastic cryotubes, and 1 ml of the cryoprotectant PGD [w/v 10% each polyethylene glycol (MW 8000), glucose and DMSO in RIB liquid medium] was added over 30 min. A further 30-min equilibration at 4 °C was followed by cooling at 0.5 °C/min (at UAD) and 0.3 °C/min (at NCGR) to −40 °C and plunging in LN. Samples were thawed for 1 min in a 45 °C water bath, transferred to a 22 °C water bath for 2 min, rinsed in liquid RIB medium and plated on RIB medium for recovery. A Cryomed 1000 freezer (Forma Scientific, Leona, MI, USA) was used at NCGR and a Planar Kryo 10 freezer (Planer Select, LTD, Sunbury, Middlesex, UK) at UAD. The Cryomed freezer was equipped with an automatic 'seeding' device that initiated ice nucleation in the cryoprotectant by quickly dropping the chamber temperature from −10 to −50 °C, then rewarming. In the case of the Planar Kryo 10 freezer, ice crystallization was induced by either exposing the cryovial manually to liquid nitrogen vapor or by touching the exterior of the vials with forceps chilled in liquid nitrogen vapor.

Vitrification

A technique modified for *Ribes* was used (Reed and Yu 1995). Meristems from cold-acclimated shoots were pretreated for 2 days under the cold acclimating conditions described above on RIB medium containing 5% DMSO (v/v). Plant vitrification solution no. 2 cryoprotectant (Yamada et al. 1991) [(v/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in liquid RIB medium with 0.4 M sucrose (RIB medium also contains glucose), at pH 5.7] was dispensed into cryotubes on ice and meristems added and stirred. After 20 min the vials were immersed in LN. Samples were rewarmed for 1 min in a 45 °C water bath and then transferred to a 22 °C water bath for 2 min.

The meristems were immediately rinsed in liquid RIB medium with 1.2 M sucrose two times, and transferred to RIB medium for recovery.

Encapsulation–dehydration

A method developed for pear (Dereuddre et al. 1990) was modified for *Ribes*. Meristems were dissected, transferred to agar plates, encased in alginate beads [3% (w/v) low viscosity alginic acid (Sigma, Poole, UK or St. Louis, USA) with 0.75 M sucrose in liquid RIB medium without calcium, pH 5.7], polymerized 20 min in calcium carbonate solution, and pretreated for 18 h in liquid RIB medium with 0.75 M sucrose. Following pretreatment, the beads were separated on sterile Petri dishes, air dried in the laminar flow hood for 4 h (approx. 20% moisture content), placed in cryotubes, and plunged into LN. Vials were rewarmed at room temperature for 15 min, encapsulated meristems were then plated on RIB recovery medium.

Experimental design and data analysis

Each cryopreservation experiment included 20 meristems distributed into 3 separate cryovials (where $n = 20$ meristems for each treatment); an additional 5–15 control (unfrozen, cryoprotected) meristems were used for each protocol. Each cryopreservation experiment was repeated three times ($n = 60$ meristems for each genotype). Assessments of the recovery of the meristems were made weekly for 6 weeks and the phenological stage reached in each case was recorded. Greening, leaf expansion, and shoot production were all required for a meristem to be considered fully recovered from the cryopreservation treatment. Data were analyzed by ANOVA and least squares means.

Results

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The average recovery of 'Red Lake' controls (cryoprotected but not frozen) was 100%, but only 10% of the frozen meristems survived following controlled freezing (0.5 °C/min) (Figure 1A). Controls exposed to PVS2 solution produced 70% regrowth for 'Red Lake' while post-cryopreservation recovery was only 30%. Control 'Red Lake' shoots exposed only to encapsulation–dehydration demonstrated 100% meristem regrowth, but after cryopreservation, only 10% grew. 'Ojebyn' meristems did not survive after controlled freezing although 95% of the controls regrew. 'Ojebyn' PVS2 controls had 85% regrowth and regrowth of 53% of the vitrified meristems. Encapsulation–dehydration produced 55% 'Ojebyn' control regrowth and 50% of cryopreserved E-D meristems regrew.

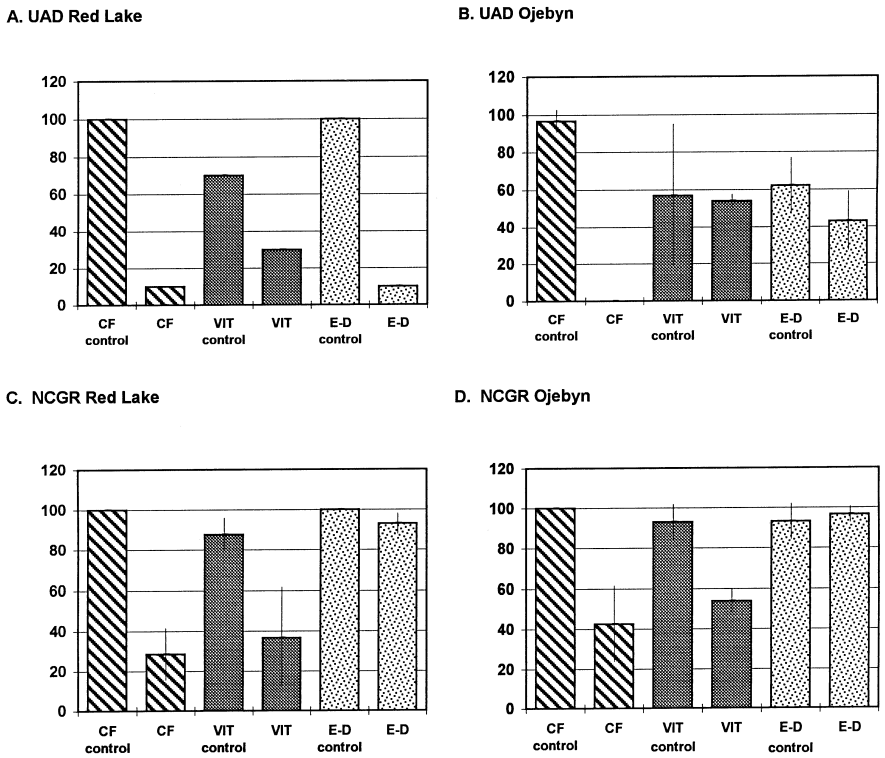

Figure 1. Regrowth percentage of meristems of *R. aureum* cv. Red Lake and *R. nigrum* cv. Ojebyn cryopreserved at The University of Abertay-Dundee (UAD) and the National Clonal Germplasm Repository (NCGR) following three protocols. Mean results of controlled freezing (CF), vitrification (VIT), encapsulation–dehydration (E–D), and controls for each are shown with standard deviations ($n = 60$). A. 'Red Lake' at UAD. B. 'Ojebyn' at UAD. C. 'Red Lake' at NCGR. D. 'Ojebyn' at NCGR.

National Clonal Germplasm Repository-Corvallis

Controlled freezing resulted in 28% regrowth of 'Red Lake' meristems with 100% regrowth for controls (Figure 1C). This was a slightly slower freezing rate (0.3 °C/min) than that used at UAD. Vitrified 'Red Lake' meristems produced 36% regrowth while controls exposed only to PVS2 recovered at 88%. Encapsulation–dehydration of 'Red Lake' meristems resulted in 93% regrowth after LN exposure and 100% for controls, but the plants had difficulty emerging from the beads. Controlled freezing (0.3 °C/min) of 'Ojebyn' produced 40% regrowth of meristems with 100% control regrowth (Figure 1D). Vitrification of 'Ojebyn' meristems resulted in 54% regrowth after LN exposure with 93% control regrowth. Encapsulation–dehydration resulted in 97% of cryopreserved 'Ojebyn' meristems regrowing and 93% control regrowth.

Discussion

When validating cryopreservation procedures it is first important to identify those variables that have the potential to determine the success of a protocol. These can be attributed to four main factors: (i) operators; (ii) plants; (iii) equipment and (iv) cryopreservation method. It is unlikely at an international level that different genebanks and laboratories will have identical culture conditions, cryogenic equipment, controlled environment facilities, and differences in operator skills will undoubtedly be an important factor. The excision of meristems from *in vitro* plants requires considerable manual dexterity, and skills differences between operators is a potential limiting factor, but one that can be overcome with training and practice. The physiological status of plant tissue cultures can greatly influence post-storage regrowth so it is essential to consider differences in *in vitro* responses (Harding et al. 1991). In this study, the same genotypes/cultivars were used in both locations, but the growth condition of the plants from which the cultures originated or the local culture conditions could have affected the final outcome. The cryopreservation equipment and protocols are the easiest factors to control. Sophisticated equipment is required only for controlled freezing. Vitrification and encapsulation–dehydration require only standard tissue culture laboratory equipment.

Method 1: Controlled rate freezing

The recovery of meristems designated as cryoprotectant controls for the controlled-freezing method was high for both locations (Figure 1). This suggests that the cryoprotectant was administered effectively without compromising the recovery of the meristems. These findings also indicate that operators in both locations were proficient in meristem dissection skills. Post-cryopreservation responses to controlled freezing results were significantly different ($P < 0.05$) between the two locations. At NCGR, 30–40% regrowth was achieved for both cultivars, while at UAD survival was 0–10% for controlled-rate freezing. This differential response can be largely attributed to differences between cryogenic equipment. The controlled-freezing method is critically dependent on the precise control of cooling rate and ice nucleation. The methodology for this protocol was first developed at NCGR using different instrumentation from that used at UAD. The NCGR instrumentation automatically initiates ice nucleation at −9 °C, the freezing point of the cryoprotectant, and samples can be cooled at a slower rate, whereas the UAD instrumentation does not function at slower rates and manual ice seeding is required. Earlier studies of three *Ribes* species comparing 0.3 and 0.5 °C/min cooling rates found no significant differences in survival due to the cooling rate, but the controlled-rate freezer had automatic seeding (Reed and Yu 1995).

Method 2: Chemical vitrification

The application of highly concentrated vitrification solutions such as PVS2 (Sakai et al. 1991) to plant tissues circumvents the need for programmable freezers. The critical factor in this protocol is stringent timing in the application and removal of the potentially toxic cryoprotectants. *Ribes* meristems (except 'Red Lake' at UAD) exposed to PVS2 solutions had regrowth not unlike those exposed to the less toxic cryoprotectants used for controlled freezing (Figure 1). The application and removal of the PVS2 solution require precise timing by the operator, but no major differences were apparent between the two locations. Post-cryopreservation regrowth was evident in both laboratories and at UAD 'Ojebyn' survival was significantly better (55%) than 'Red Lake' (30%) while recoveries at NCGR were not statistically different. There were no significant differences ($P < 0.05$) in regrowth between the two locations. A range of survival of 10–70% is noted for vitrified *Ribes* meristems in several studies with many genotypes reaching 40–70% regrowth (Reed and Yu 1995; Benson et al. 1996; Luo and Reed 1997). These results may be further improved if recent advances in pretreatment, which improve the response of cells to the vitrification solution, are incorporated into the protocols (Luo and Reed 1997).

Method 3: Encapsulation–dehydration

Non-cryopreserved, E-D control meristems had 95–100% regrowth except for 55% regrowth of 'Ojebyn' at UAD (Figure 1). The encapsulation–dehydration method is critically dependent upon the optimization of dehydration and meristem desiccation tolerance is an important survival factor. Our results may reflect differences in the physiological status of the cultures in response to desiccation or culture conditions. This idea is further endorsed by the cryopreservation results as encapsulation–dehydration produced excellent regrowth for both genotypes at NCGR and good results for 'Ojebyn' at UAD. In contrast, regrowth after cryopreservation for 'Red Lake' was poor at UAD, despite the fact that this cultivar had excellent control regrowth following desiccation. A previous study at UAD found good post-cryopreservation recovery (70–80%) for *R. nigrum* cvs. Ben Tron and Ben More using encapsulation–dehydration (Benson et al. 1996). It is possible that the poor response of 'Red Lake' at UAD in the present study was due to physiological and/or cultural factors that could be overcome by further optimization of the culture conditions or desiccation treatments for this species as was seen in grass meristems (Chang 1999; Chang et al. 2000).

Overall trends from this study indicate that 'Ojebyn' responds better to cryopreservation than 'Red Lake' at UAD, but regrowth of the two genotypes was similar at NCGR. Regrowth of the two genotypes following any of the individual cryopreservation protocols was not significantly different at NCGR, but significant differences were apparent between the genotypes at UAD. Plant vitrification solution no. 2

vitrification and encapsulation–dehydration produced similar (40–60%) regrowth of 'Ojebyn' at UAD, whereas encapsulation–dehydration was significantly more effective (95%) for this genotype at NCGR. Overall, 'Red Lake' performed poorly after cryopreservation at UAD and this may be due to the fact that this culture was particularly slow growing under standard culture as compared to 'Ojebyn' (R.M. Brennan, personal communication).

In developing cryopreservation methods for temperate soft fruit germplasm it is important to consider the physiological status of the culture prior to conserving and for this reason cold acclimation is an important component of *Ribes* protocols. In this study, the facilities used to achieve cold acclimation were somewhat different; at NCGR cold acclimation was 8-h light at 22 °C and 16-h dark at −1 °C while UAD was 8-h light at 25 °C and 16-h dark at 4 °C. However, the small difference in the temperature probably had little overall effect on the resulting level of cold acclimation. The most important factor influencing cold acclimation of *in vitro*-grown pear shoots is the use of alternating temperature treatments and most temperate plants probably have a similar response (Chang 1999; Chang and Reed 2000).

In conclusion, variable responses are evident between the two laboratories for two of the three techniques, largely due to differences between cryogenic equipment and differences in the physiological status of the cultures. Once these differences were identified changes could be made to improve recovery. Careful attention to the details of protocols often makes the difference between success and failure when using procedures developed in other laboratories. The physiological condition of the stock plants from which meristems are taken has a major bearing on cryopreservation results (Harding et al. 1991) and growth room conditions at each facility are unique (Reed 1996; Wu et al. 1999). Thus, it is not surprising that the transfer of cryopreservation protocols from one laboratory to another may result in variable responses. Work is now underway to apply these methods to a wider range of germplasm of the genus *Ribes* at both NCGR and UAD.

Our recommendations for future validation studies are the precise reporting of all details of protocols as this will be necessary to standardize the procedures. In addition researchers must identify points where significant difficulties may arise in the procedure. As much of the success of tissue culture and cryopreservation protocols is dependent on familiarity with the procedures, direct 'hands on' training is advised as this is more effective than written instructions due to the possibility of different interpretations. The protocols used in this study were developed for other genera, then successfully adapted to the genus *Ribes* (Dereuddre et al. 1990; Reed 1990; Yamada et al. 1991). Our studies indicate that some protocols are more easily transferred than are others, and success with a protocol can be linked to specific portions of each process. The three methods in this study produced different results in the two laboratories, however the subsequent modification of techniques in both NCGR (Luo and Reed 1997) and UAD (Dumet et al. 2000) improved post-cryopreservation regrowth responses considerably.

Finally, as recent breakthroughs in cryoprotective methodologies lead to the wider application of cryopreservation in plant genetic resources management it will become increasingly important to address technology transfer and methods validation issues. To achieve this aim, effective networking and collaboration between different repositories and cryopreservation facilities will be essential.

Acknowledgements

Partial funding for this collaboration from NATO (grant no. CRG 940164) is gratefully acknowledged, as is technical support from Isobel Pimberley and Mike Black (Abertay). Work at NCGR-Corvallis was supported by CRIS 5358-21000-014. Rex Brennan (Scottish Crop Research Institute, Dundee) is thanked for providing *Ribes* germplasm.

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