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Evaluation of Critical Points in Technology Transfer of Cryopreservation Protocols to International Plant Conservation Laboratories

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Abstract

Cryopreservation of plant tissues in liquid nitrogen is now used for long-term conservation of vegetatively-propagated crops. Development of standard techniques for cryopreservation is important to the international plant-conservation community for successful implementation of storage protocols in diverse and internationally dispersed laboratories. Evaluation of the critical points of each preservation technique will greatly assist in developing and validating internationally-used cryopreservation protocols. The goals of this project were to assess critical points of two major cryopreservation techniques (PVS2 vitrification and encapsulation dehydration) during their transfer to international laboratories; analyze post-storage viability for each technique and location; and develop recommendations based on the assessments and data from the participating laboratories. Investigators from Germany, Kazakhstan, Poland and UK participated in a 2-week training workshop in cryopreservation methods after which the techniques were tested in the home laboratories of the participants. After one year site visits by the technology trainers identified critical points in the protocols. Critical points were identified as 1) Cryogenic (cryoprotection, LN exposure, rewarming); 2) Non-cryogenic (plant health status, pre- and post-storage culture); 3) Operational (skills transfer, training, interpretation of procedures); 4) Facility (growth room, ambient conditions, media preparation, equipment). The most critical factors in all laboratories were culture health, operator skills and experience, and clarification of the technical details of the procedures. Final results showed that correction of critical factors improved the post-storage recovery in all the laboratories.

Keywords: Meristem, shoot tip, *Ribes*, liquid nitrogen, germplasm

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 modern technologies with traditional conservation strategies is important for the successful preservation of plant biodiversity (3, 6, 7). Traditionally, plant genetic resource management involves conserving germplasm as seed at low temperatures, 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. One of their main advantages is that they offer added security for field genebank conservation (1). The ideal genetic-resource conservation program consists of active collections that are readily 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 establish and cryopreservation is now considered the most appropriate option for these systems (1, 2).

A report from the International Plant Genetic Resources Institute highlights the role of *in vitro* conservation methods in germplasm storage (1). This suggests that it will become increasingly important to validate new storage protocols, especially those that employ cryogenic methods at the international level. As *in vitro* conservation comprises two interdependent techniques, tissue culture and cryogenic storage, it is important that validations consider both aspects. Cryopreservation is the storage of living cells and tissues in liquid nitrogen (LN) at ultra-low temperatures (-196^{o}C) and is now applied to a diverse range of plant species and tissue systems (1, 2, 3). The development of many cryoprotection and cryopreservation methods (4, 15) 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 colligative-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 (-30 ^{o}C to -50 ^{o}C). On reaching this point the tissues are transferred to liquid nitrogen. Controlled freezing can be varied in many ways (10, 16, 24, 26). The precise control of cooling rates, colligative cryoprotectants, and extracellular ice nucleation is critical to the success of cryopreservation using controlled-freezing methods and can be most reliably achieved using programmable controlled-rate freezers. Vitrification is the second approach to cryopreservation and involves the application of cryoprotective treatments that increase cell viscosity to a critical point at which water forms an amorphous, metastable, glassy state on exposure to ultra-low temperatures (13, 22, 25). 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 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 employs vitrification through the application of osmotic and evaporative dehydration (8, 9, 14). Plant tissues are encapsulated in alginate beads and exposed to sucrose loading followed by desiccation in either a sterile air flow or over silica gel to a critical moisture level. For either vitrification approach, the tissues are directly plunged into liquid nitrogen and the water molecules vitrify.

Validation and technology transfer of established and new protocols for use among international genebanks is important for the integration of cryopreservation into traditional plant genetic resource conservation systems. It is important to develop reliable and reproducible

methodologies that can be applied across a broad genotype range and that can be routinely implemented by different repositories (18). It is especially important to ensure that plant cryopreservation methodologies are transferable to laboratories throughout the world. Good-practice procedures are now available for internationally designated microbial and animal-cell 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 (23).

Techniques for liquid nitrogen storage of clonally propagated plant germplasm are now widely available (1, 2, 3), but are not routinely used in genebanks (20). For the wider adoption of cryogenic storage in genebanks it is essential to validate cryopreservation protocols in independent laboratories in several countries (21). Such an approach will aid the successful implementation of cryo-conservation methodologies in plant genebanks worldwide. In an earlier study, members of the project team validated three cryopreservation techniques: controlled freezing, PVS2 vitrification and encapsulation dehydration in genetic resources laboratories based in two locations: the USDA-ARS National Clonal Germplasm Repository (NCGR), Corvallis, Oregon, USA and the University of Abertay Dundee (UAD), Scotland (5, 21). The objectives of the current study were to develop a critical-point assessment of the two vitrification protocols; transfer the techniques to additional laboratories; analyze storage viability for each protocol and location based on critical-point assessments; and develop recommendations for the facilitation of technology transfer based on the critical-point assessment and data from the participating laboratories. Critical points were identified as 1) Cryogenic (cryoprotection, LN exposure, rewarming); 2) Non-cryogenic (plant health status, pre- and post-storage culture); 3) Operational (skills transfer, training, interpretation of procedures); 4) Facility (growth room, ambient conditions, media preparation, equipment). The goals of this study were to identify those components of cryopreservation protocol development and technology transfer that critically influence the successful implementation of cryo-conservation methodologies in international plant conservation laboratories.

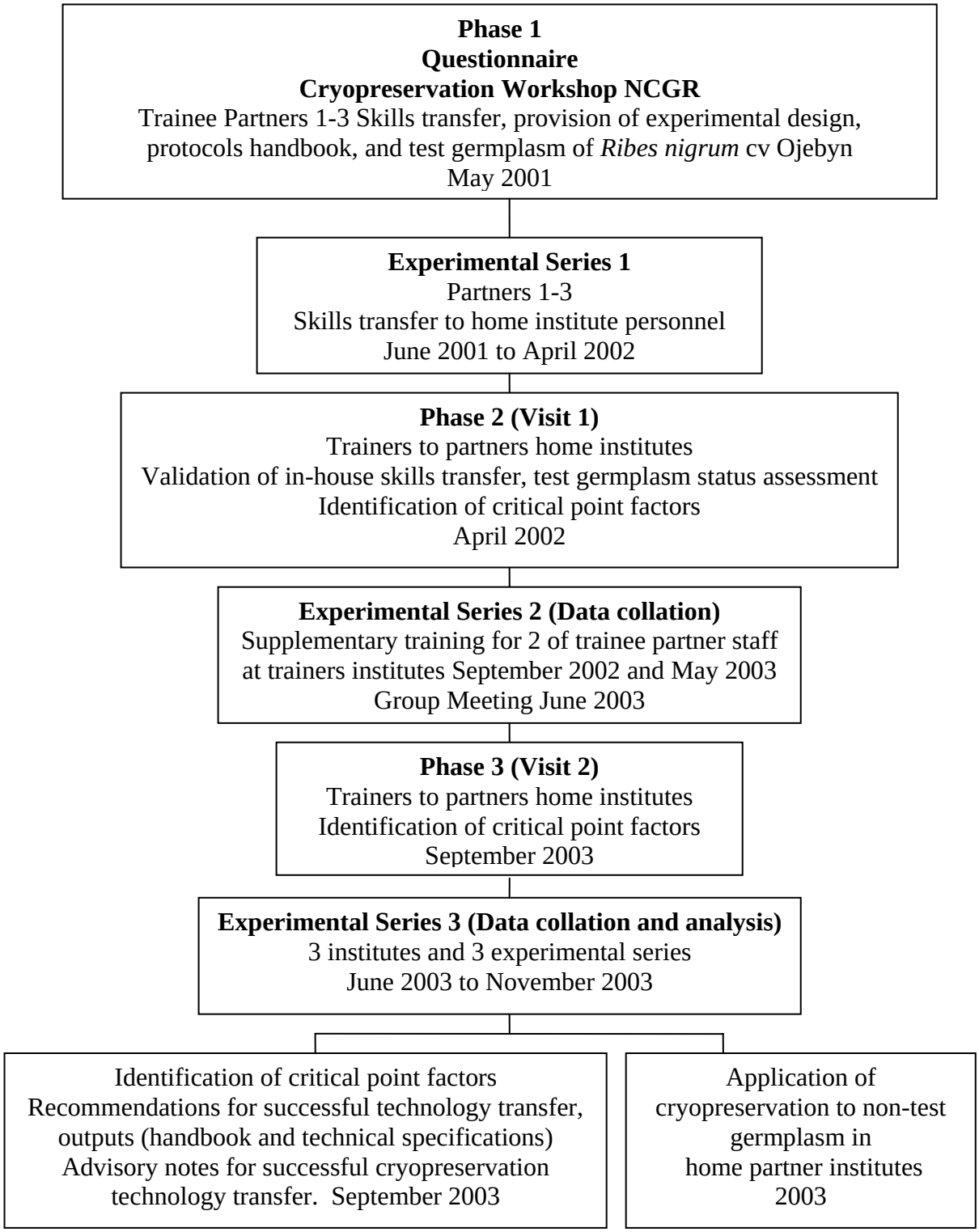
MATERIALS AND METHODS

Technology transfer:

Initial training and laboratory status evaluation: The collaborative team was composed of investigators from laboratories in Germany, Kazakhstan, Poland, UK and USA. Three laboratories were selected to undertake the training and experimental processes, and undergo assessment of the critical-point factors and the efficiency of cryopreservation technology transfer. Principal investigators from these laboratories were trained in PVS2-vitrification and encapsulation-dehydration cryopreservation techniques during a 2-week workshop in May 2001 at the National Clonal Germplasm Repository (NCGR), Corvallis, Oregon. Each scientist was given a cryopreservation manual and a set of experiments to perform in their home laboratories (see below). Partners completed a questionnaire to document facility profile and operator skills experience before the onset of the project.

Figure 1

Organizational Structure and Events Sequence for Critical-Point Assessments of Cryopreservation Methodology Technology Transfer



First critical-point assessment: In April 2002, the partner laboratories were visited (Figure 1) to determine how effectively the techniques were being implemented and to ascertain what difficulties were involved in transferring the conservation technologies in each facility. Visits consisted of about 5 days at each laboratory and included assessing the facilities (particularly ambient conditions that affected temperature regulation and relative humidity); viewing the operators as they excised shoot tips using aseptic dissections, prepared solutions, and worked through the protocol procedures. Critical points were evaluated and corrective recommendations made (Table 1). Data (regrowth of control and cryopreserved shoot tips six weeks after thawing) was collected from the first set of experiments at each laboratory.

Table1. Critical points for assessment of transfer of major cryopreservation techniques

Personnel • Basic laboratory skills • Tissue culture expertise • Dissecting skill • Stringency to protocol procedures and thoroughness • Success in validation of protocol	**Culture Conditions** • Standard culture regime • Growth room parameters • Culture medium • Growth regulators • Subculture interval
Source Plant Status • Origin • Time in culture • Subculture transfer interval • Genotype 'Ojebyn'	**Pregrowth and Recovery** • Acclimation • Pregrowth pretreatment • Medium for growth and recovery • Growth room parameters • Subculture intervals
Step by Step Instructions • Cryoprotectant solution preparation • Meristem dissection • Vitrification protocol • Encapsulation-dehydration protocol • Controls for cryopreservation and LN handling • Rewarming and rehydration • Rinsing and plating	**Cryogenic Facilities** • Type of dewar • Vials, canes, boxes • Inventory system • Labeling • Nitrogen availability
General Facilities • Growth room • Laminar-flow benches • Water bath • Cold-hardening facilities • General laboratory facilities	

Group meeting: Each laboratory repeated the experiments (Set 2) taking into account the recommendations provided after analysis of the first experimental dataset (May 2002 to May 2003). The group subsequently met to discuss technology transfer developments throughout the different project teams in June 2003. This meeting identified critical-point issues related to protocol technology transfer (technical, operational and laboratory capability) and how these affected development of laboratory cryogenic storage. Discussions highlighted actions required to ameliorate technology transfer difficulties.

Additional training: Scientists from 2 of the 3 laboratories received additional training in the partner laboratories following the second set of experiments (Figure 1).

Second critical-point assessment: Experiments were repeated (Set 3) in the summer of 2003 and a second critical-point evaluation (Figure 1) was undertaken in September 2003. Critical points were evaluated and final recommendations made for the future use of cryopreservation techniques in the participating laboratories.

Experimental procedures:

Micropropagated shoots of *Ribes nigrum* L. cv. Ojebyn obtained from NCGR-Corvallis, were multiplied and shoot tips recovered on NCGR-*Ribes* medium (RIB), which contains the mineral salts and vitamins of Murashige and Skoog (12) 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 at 25°C with a 16-h light (25 µmol•m^{-2}•s^{-1})/ 8-h dark photoperiod. Cold acclimation was 8-h light at 22°C and 16-h dark at -1°C. All cultures were cold acclimated for 1 week (17). After acclimation 0.8 mm apical shoot tips were excised for cryopreservation.

PVS2 vitrification: A technique modified for *Ribes* was used (11, 19). Shoot tips from cold-acclimated shoots were pretreated for 2 days under the cold-acclimating conditions described above on MS medium containing 5% DMSO (v/v). Shoot tips were pretreated for 2 h on ice or in the refrigerator in 1% (w/v) Bovine Serum Albumen (BSA) mixed in 0.4 M sucrose MS solution. Then the BSA was removed and PVS2 cryoprotectant (27) [(v/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in liquid MS medium with 0.4 M sucrose, at pH 5.7] was dispensed into cryotubes on ice and shoot tips 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 shoot tips were immediately rinsed twice in liquid MS medium containing 1.2 M sucrose, and transferred to RIB medium for recovery.

Encapsulation-dehydration: A method developed for pear (8) was modified for *Ribes*. Shoot tips 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 MS medium without calcium, pH 5.7], polymerized 20 min in 100 mM calcium chloride solution, and pretreated for 18 h in liquid MS 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 on a fresh weight basis), placed in cryotubes, and plunged into LN. Vials were rewarmed at room temperature for 15 min, rehydrated in liquid medium for 5 min, and encapsulated shoot tips were then plated on RIB recovery medium with 5 g/L agar to aid in rehydration of the beads.

Experimental design and data analysis: Each cryopreservation experiment was performed by the 3 laboratories and each experiment included 30 shoot tips distributed into 3 separate cryovials (where n = 30 shoot tips for each treatment); an additional 5-15 control (cryoprotected, not frozen) shoot tips were used for each protocol. Each cryopreservation experiment was repeated three times (n = 90). Assessment of the recovery of the shoot tips was made at 6 weeks. Greening, leaf expansion, and shoot production were all required for a meristem to be considered fully recovered from the cryopreservation treatment. Each experiment was performed 3 times for a "Set". In most cases Set 1 was performed following the training workshop (Figure 1) June 2001 to April 2002; Set 2 occurred following the laboratory visit May 2002 to May 2003; Set 3 occurred following the group meeting June 2003 to November 2003.

RESULTS AND DISCUSSION

Technology transfer:

Initial training: The two-week initial training provided familiarity with the techniques, but was not long enough to allow the participants to become proficient with each step. In this instance the trained individuals returned to their laboratories and taught the techniques to laboratory personnel who performed the actual experiments. Participants suggested that additional repetitions of the experiments during the training period might have circumvented some of the errors related to steps of the protocols and interpretation of instructions related to solution preparation. Some additions and clarifications to the manual were thus required to effectively implement technology transfer instructions compromised by potentially ambiguous explanations lost on translation. Translation of experimental steps into partner languages was best done at this point so that the finer technical points of the procedures (particularly related to cryoprotectant preparation) are clarified.

Questionnaire: The questionnaire provided baseline information about each laboratory and allowed for an initial comparison of facilities, personnel and research background.

First critical-point assessment: A number of points were critical in each of the partner laboratories (Table 1), but each was unique in the different protocol and operational components that needed attention. The first critical points were non-cryogenic. Expert dissection of shoot tips was crucial to regrowth of control and cryopreserved shoots. Practice in dissection of shoot tips should result in 80-100% of dissected tips developing into plants. Other non-cryogenic factors were the health of the plant cultures, and the condition of the growth facilities.

The second group of critical points involved operational aspects of the protocols. Solution preparation and storage were dependent on clear presentation of the procedures in the manual and the precise, non-ambiguous translation of the manual into additional languages from English by the partner scientists. For example, some steps of the protocols were either difficult to describe in words or meaning was lost on translation and this required physical demonstrations of the correct procedures.

Facility issues were found to be important across all partners and particularly related to the variation in functioning of laminar flow hoods and seasonal fluctuations in building heating and cooling systems. The encapsulation-dehydration technique was particularly influenced by ambient environmental parameters (humidity, temperature) and interference

with evaporative desiccation of alginate was a major critical factor in some cases. No critical problems with cryogenic factors were noted.

Group meeting: The group held discussions (Figure 1) of critical points from the second set of experiments and the project in general. All laboratories indicated that standardizing the alginate-bead drying process was one of the most difficult parameters to control. Room temperatures and humidities had a wide range of seasonal variations and the capacity to control environmental parameters was influenced by the type, if any, of the regulatory equipment used. It was recommended that for the implementation of future studies each laboratory standardize the evaporative desiccation by using a known amount of activated silica gel in a closed desiccator, preferably placed in a controlled temperature room.

Problems related to secondary levels of skills transfer were also identified. Technology transfer was initiated via the training of supervisors who then passed on their newly acquired knowledge and skills to home laboratory personnel. Follow up training in participant institutes identified the need for primary training to also be received by laboratory personnel in order to avoid problems associated with technical and theoretical skills uptake. Participants felt that performing further experiments during the training and scheduling the initial laboratory visits at 6 months would improve the technology-transfer process. Each laboratory presented plans for future cryopreservation projects as applied to a wider species base.

Second critical-point assessment: The second laboratory visit involved a comparative evaluation of critical-point factors, procedures and draft compilations of data sets obtained for each of the three experimental phases (Table 1). At this stage cryogenic and non-cryogenic critical points were successfully resolved, but operational factors remained limiting factors. These were mostly related to the clarity of the instruction manual and varying interpretations of cryoprotectant solution preparation. These points highlight the need for field-testing of manuals and paying stringent attention to language and translation. Critical-point facility concerns included variation among autoclaves, laminar flow bench performance, and laboratory heating and cooling systems. These facility differences require local corrective action or the application of alternative strategies for optimal experimental success.

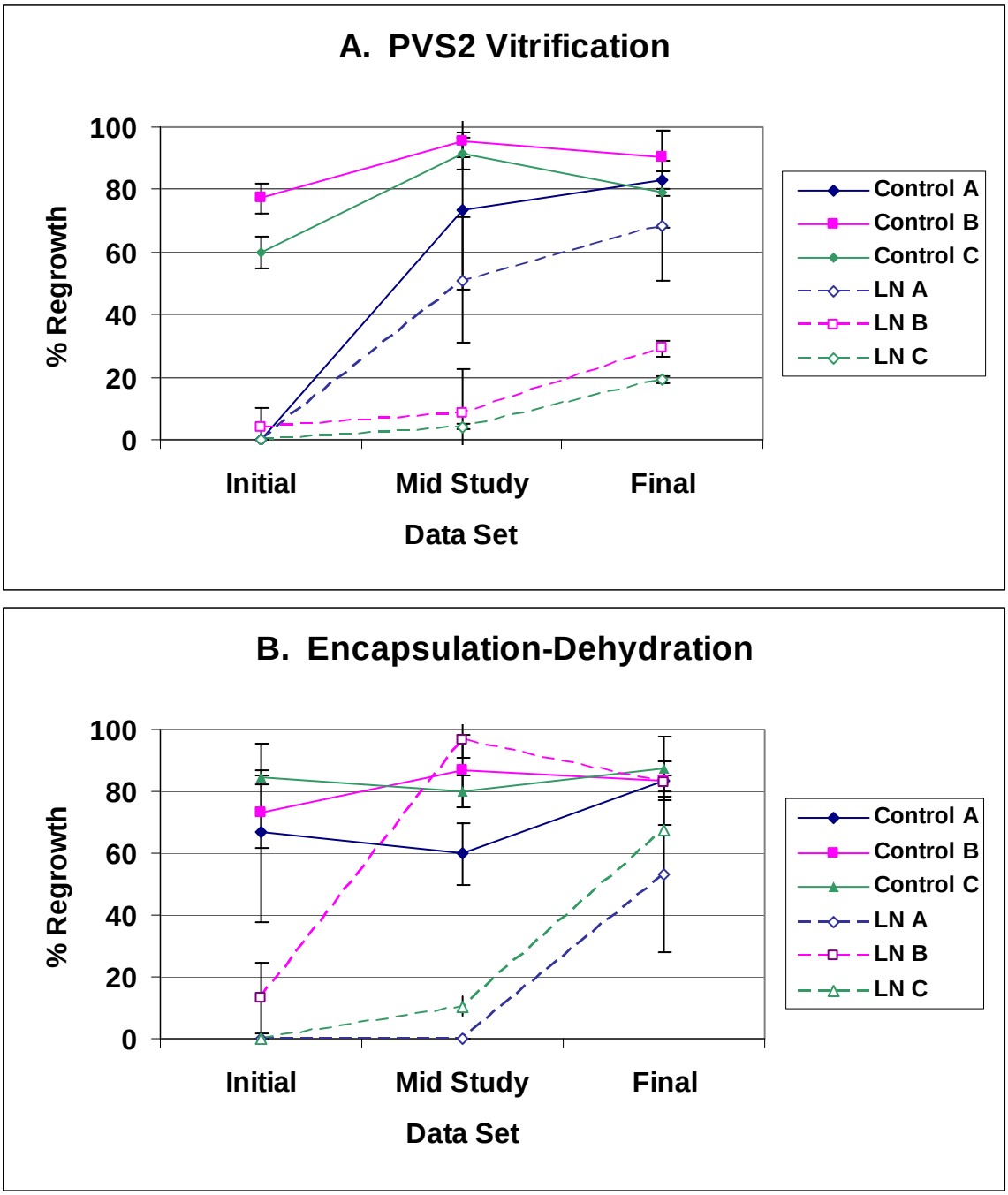
Experimental Data:

Initial experiments: The first experiments (Figure 2) were generally unsuccessful due to the critical points identified in Table 2. **Vitrification** (Figure 2A): Observation of control shoot-tip recovery indicated that shoot-tip dissection procedures limited recovery in Laboratory A. In comparison, Laboratory B identified that key environmental parameters were the most important factors to affect survival, and in the case of Laboratory C misinterpretation of steps in the procedures resulted in lack of recovery of shoot tips. **E-D** (Figure 2B): In all three laboratories variations among laminar flow and room temperatures affected drying times and resulted in little or no recovery of shoot tips following E-D and cryopreservation.

Table 2: Categories and critical points identified at two assessment times during cryopreservation technology transfer experiments and suggested corrective actions

Assessment & Time	Critical Point	Corrective Action
Facility 1	Sub-optimal rates of evapo -desiccation due to low laminar air flow	Adjust air flow; explore use of silica gel for desiccation
Non-cryogenic 1	Absence of regrowth of in control shoot tips	Increase dissection practice and prevent desiccation injury during excision by performing manipulations on moistened sterile filter papers. Achieve 80-100% regrowth before commencing cryogenic procedures
Non-cryogenic 1	Sub-optimal growth of donor plants leading to poor recovery in controls and cryopreserved shoots	Identify factors causing tissue culture problems: sub-optimal control of culture room regimes
Operational 1	Alginate beads are irregular in shape and size	Dispense alginate solutions into excess calcium chloride using a vertically aligned pipette to ensure spherical uniformity. Routinely use the same size pipette
Operational 1	No regrowth following E-D and LN following all periods of dehydration tested; beads have a moisture content of >20% fresh weight basis	Calibrate the time required to reach 20% percent bead moisture against ambient laboratory environmental parameters (temperature RH) achieved during evaporative desiccation. In the event that this cannot be controlled use silica gel
Operational 1,2	BSA and PVS2 solutions prepared incorrectly leading to PVS2 crystallizing on contact with LN	Clarify instructions in the manual particularly translations for accuracy in describing to numerical calculations. These aspects will not have an effect on the physical properties of vitrification solutions but they will influence cryoprotectant toxicity (see below)
Operational 1,2	Cryoprotectant toxicity	Use high purity cryoprotectants, (particularly DMSO), filter sterilize and, use of the day of preparation
Facility 2	Caramelization of autoclaved sucrose solutions	Check length of autoclave cycle; filter sterilize solutions if cycle length cannot be reduced
Facility 2	Seasonally fluctuations in laboratories leading to excessive temperatures	Add air-conditioning unit to laboratory; record temperature and RH during operations
Non-cryogenic 2	Seasonal microbial contamination in recovering shoot tips	Apply stringent visual checking of cultures for bacterial and fungal contaminants; test autoclave and filter sterilization procedures. If possible install air conditioning to circumvent venting via open windows; exercise clean lab procedures
Operational 2	Plantlets do not grow out of the alginate beads	Rehydrate the alginate beads in MS medium for 5 min before plating on the recovery medium

Figure 2: Percent regrowth of shoot tips from 3 replications of (A) PVS2 Vitrification or (B) Encapsulation-Dehydration in each laboratory (A, B, C) at three stages in the program. (Percent regrowth ± standard deviation) for control and liquid nitrogen exposed (LN) shoot tips.



Mid-study experiments: The second set of experiments resulted in improved recovery in most cases. **Vitrification** (Figure 2A): The vitrification technique was the most difficult to implement through technology transfer. Although control recovery was high, cryopreserved shoot-tip recovery remained lower than expected for two laboratories. In Laboratory A improved shoot-tip dissection and additional training in a training-partner laboratory led to improved results. **E-D** (Figure 2B): Successful calibration of drying times in Laboratory B resulted in excellent regrowth of cryopreserved shoot tips. Laboratories A and C had difficulties with solution preparation and/or drying.

Final experiments: Many of the critical points determined in earlier experiments were resolved and each group gained experience with the techniques during the first experimental sets. **Vitrification** (Figure 2A): Laboratory A improved dissection skills and further improvements were made by using filter sterilization of some of the solutions in preference to long-hold autoclaving which caused caramelization and possible breakdown of cryoprotectants. Progress in Laboratory B was hampered by high ambient room temperatures during this experimental set which possibly compromised cryoprotectant stability. Laboratory C needed to resolve some difficulties in cryoprotectant solution preparation. **E-D** (Figure 2B): E-D results improved greatly with standardization of bead desiccation in all laboratories.

Conclusions

This project reports for the first time, the identification of technical, operational and practical issues associated with the transfer of plant cryopreservation protocols to dispersed international laboratories. Importantly a number of common, critical-point factors were identified through reciprocal visits and bi-lateral feedback discussions. These required corrective actions to both trainer and trainee components of the skills transfer process. As a result it was possible to assess those parts of the cryopreservation protocol development and technology transfer that were most likely to restrict the potential for optimal skills competency. Subsequent stringent and detailed attention to critical-point factors resulted in the successful implementation of cryopreservation techniques in follow up experiments. As many of these factors will be common in plant culture laboratories (18), it is anticipated that this study will assist practitioners in other sectors that are planning to embark on the establishment of new cryogenic facilities and implementation of cryogenic techniques.

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