Breeding Science 65: 41–52 (2015)
doi:10.1270/jsbbs.65.41

Review

Cryopreservation for preservation of potato genetic resources

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Cryopreservation is becoming a very important tool for the long-term storage of plant genetic resources and efficient cryopreservation protocols have been developed for a large number of plant species. Practical procedures, developed using *in vitro* tissue culture, can be a simple and reliable preservation option of potato genetic resources rather than maintaining by vegetative propagation in genebanks due their allogamous nature. Cryopreserved materials insure a long-term backup of field collections against loss of plant germplasm. Occurrence of genetic variation, in tissue culture cells during prolonged subcultures, can be avoided with suitable cryopreservation protocols that provide high regrowth, leading and facilitating a systematic and strategic cryo-banking of plant genetic resources. Cryopreservation protocols for potato reviewed here, can efficiently complement field and *in vitro* conservation, providing for preservation of genotypes difficult to preserve by other methods, wild types and other species decided as priority collections.

Key Words: cryopreservation, D cryo-plate, DMSO droplet, droplet vitrification, encapsulation vitrification, potato genetic resource, V cryo-plate.

Introduction

Cryopreservation is becoming an increasingly used method for the long-term storage of plant genetic resources (PGRs). Cryopreservation requires only a minimum of space and low level of maintenance. Methods for cryopreservation have been developed for a large number of plant species and further research is being conducted to enable adoption of this approach even more broadly (Li and Pritchard 2009, Pritchard *et al.* 2013). A timely book 'Plant Cryopreservation—A Practical Guide' was published to aid in the use of cryopreservation techniques globally, for the preservation of all forms of plant biodiversity (Reed 2008). Also, an updated 'Genebank Standards for Plant Genetic Resources for Food and Agriculture' was issued from Commission on Genetic Resources for Food and Agriculture (FAO 2014 www.fao.org/docrep/meeting/027/mf804e.pdf). In this book, one chapter discusses genebank requirements for *in vitro* culture and cryopreservation. Thus cryopreservation techniques using *in vitro* shoot tips are recognized as a long-term storage tool for PGR.

Recent research on cryopreservation has focused on practical procedures for genebank storage, thereby enabling cells and meristems to be cryopreserved by direct transfer into liquid nitrogen (LN). The development of simple and reliable methods for cryopreservation facilitates cryo-banking. Optimal cryopreservation conditions produced high levels of regrowth after LN storage. Potato is one of the most important food crops for food security. There are more than 4,500 varieties of *Solanum tuberosum* L. (Hils and Pieterse 2009). Preservation of potato genetic resources (GRs) in genebanks is mostly by vegetative propagation due their allogamous nature and many genebanks are maintaining potato GRs as field collections.

A review by Kaczmarezyk *et al.* (2011) discussed in detail recent advances in potato cryopreservation based on thermal, biochemical, genomic and ultrastructural analyses. There are some recent reviews of the development of potato cryopreservation protocols (Gonzalez-Amano *et al.* 2008, Keller *et al.* 2008, Wang *et al.* 2008). This review introduces practical and successful cryopreservation protocols of *in vitro* grown potato shoot tips, which have been used and are being implemented for cryo-banking in institutions around the world. In addition, here new cryopreservation techniques using aluminium plates developed by Yamamoto *et al.* (2011b) and Niino *et al.* (2013) are described and the importance of cryopreservation techniques for long-term storage are discussed.

Ex situ preservation

Ex situ preservation of PGRs is the storage of seeds or plant materials under artificial conditions to maintain their long-term viability and availability for use. Globally, genebanks

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Communicated by K. Watanabe
Received September 5, 2014. Accepted October 22, 2014.
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are employing seed storage, field collections, *in vitro* storage (tissue culture or cryopreservation) for ex situ preservation of PGR. Storage of PGR that produce orthodox seeds, which are tolerant to low moisture content and low temperatures, at appropriate temperature and humidity, is the most convenient *ex situ* preservation method. Many major seed crops are included this category. However, recalcitrant seeds, which are sensitive to low moisture content and low temperatures, do not survive if they are stored under the standard storage conditions used for orthodox seeds. This category of seeds includes several important tropical and sub-tropical tree species. There is one more category recognized as intermediates between orthodox and recalcitrant seeds and known as intermediate seeds, which can tolerate combinations of desiccation and low temperature.

Field genebanks maintain living plants. Field genebanks are used for the plants which produce non-orthodox seeds or no seeds and are vegetatively propagated. Vegetatively propagated plants comprise of two types, the perennial and annual/biennial types. The former can be maintained in the field for long period without replanting but for the latter replanting is necessary annually or biennially. The preservation of these PGRs requires an adequate area of land and continuous maintenance. Vegetatively maintained PGRs are vulnerable to loss from natural disasters and damage caused by pests and diseases.

In vitro genebanks are a means to overcome the disadvantage of the field genebanks and reflect progress in plant tissue culture techniques. Preserved *in vitro* germplasm can be propagated and regenerated into plantlets in a sterile and pathogen free environment. *In vitro* genebanks are used for species with an established tissue culture system. To maintain *in vitro* germplasm, it should be subcultured after specific periods of time under standard culture conditions to avoid deterioration and/or contamination of materials. Several slow growth (minimal growth) methods have been established for short (3 months) to middle (3 years) term storage using low temperature, minimal nutrition, growth retardant and so on, singularly or in combination (Oka and Niino 1997). A drawback of tissue culture storage is the induction of genetic variation or mutation during prolonged subculturing. For this reason, minimal growth method is desirable for preservation of *in vitro* materials reduce the subculture interval. Selection of explant is also important in *in vitro* storage as somaclonal variation from cultured cells and callus may occur more easily compared to *in vitro* shoot cultures. Hence there is a preference for using shoots for *in vitro* storage by minimal growth method. Details regarding preservation methods can be found at: http://cropgenebank.sgrp.cgiar.org/index.php/procedures-mainmenu-243/conservation-mainmenu-198.

In vitro genebanks of potato

In vitro potato collection of 1,223 accessions are maintained at 25°C in the National Institute of Crop Science (NICS), Rural Development Administration (RDA), Rep. Korea (Kim personal communication 2014). Also, in the National Center for Seeds and Seedlings (NCSS), Japan, an *in vitro* potato collection of 130 accessions is maintained at 25°C and normal subculture by node or shoot is performed every 3–4 months. The frequency of subculturing can be reduced by growing the micro plants on Murashige and Skoog medium (MS medium, Murashige and Skoog 1962) supplemented with growth retardants or osmotic stress inducing polyols, incubating them under low temperature, low light intensity and varied photoperiod (Gopal and Chauhan 2010). The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany maintains 2,855 potato accessions *in vitro* at 4°C as microtubers. The cycle of slow growth maintenance consists of a warm phase with long-day at 20°C for 2–3 months, a microtuber induction phase with short-day at 9°C for 2–4 months and a cold storage period, in which microtubers are stored at 4°C for 12–15 months (Keller *et al.* 2006, Keller personal communication 2014). At the Central Potato Research Institute, Shimla, India, more than 1,500 parental lines and potato varieties are maintained *in vitro* on MS medium supplemented with 40 g/l sucrose and 20 g/l mannitol at 6–8°C and 16-h photoperiod (Gopal and Chauhan 2010). At the International Potato Center (CIP), Peru, the *in vitro* collection consists of 4,062 accessions that are maintained under slow growth conditions. The medium used contains MS salts, 40 g/l sorbitol, 20 g/l sucrose, 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.4 mg/l thiamine and 8 g/l agar. Cultures are maintained at 6–8°C under 22 μmol/m^{2}s illumination and 16-h light. This allows *in vitro* plantlets to be stored for approximately 2 years without sub-culturing (Panta personal communication 2014). Besides these Institutes, *in vitro* storage of potato GRs is conducted at many other Institutes, such as National Forestry, Crops and Livestock Research Institute, Mexico and National Institute for Agricultural Research, Chile. Almost all Institutes mentioned above have started research on cryopreservation of potato GRs as an alternate of *in vitro* storage.

The concept of vitrification

Cryopreservation is based on the reduction and subsequent interruption of metabolic functions of biological materials by decreasing the temperature with LN (–196°C), while maintaining viability. At –196°C, almost all the cellular metabolic activities are quiescent and the cells can be preserved in such state for a long-term. It is essential to avoid lethal intracellular freezing that occurs during rapid cooling in LN and warming in order to maintain the viability of hydrated cells and tissues (Sakai and Yoshida 1967). Cells and tissues that are to be cryopreserved in LN, need to be sufficiently dehydrated before being immersed in LN.

There are two types of liquid-solid phase transitions in aqueous solutions. (a) Ice formation is the phase transition from liquid to ice crystals, and (b) vitrification is a phase transition from a liquid to amorphous glass that avoids crystallization (Sakai *et al.* 2008). Water is very difficult to vitrify because the growth rate of crystals is very fast, even just below freezing point. However, highly concentrated cryoprotective solutions such as glycerol are very viscous and are easily supercooled below –70°C. This allows them to be vitrified on rapid cooling (Sakai 1997, Sakai *et al.* 2008). Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass without crystallization (Fahy *et al.* 1984). Vitrification had been proposed as a method for the cryopreservation of biological materials because of the potentially detrimental effects of extracellular and intracellular freezing might be avoided (Luyet 1937). Thus, vitrification is an effective freeze-avoidance mechanism for hydrated cells and tissues. As glass fills space in a tissue, it may contribute to the prevention of additional tissue collapse, solute concentration, and pH alteration during dehydration. Operationally, glass is expected to exhibit a lower water vapor pressure than the corresponding crystalline solid, thereby preventing further dehydration. Because glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to dormancy and stability over time (Burke 1986). In any cryopreservation method, whole specimens or partial parts of specimens, which are in sufficient concentration of cytosol, can vitrify by rapid cooling into LN. In the plant vitrification method, plant vitrification solution (PVS) is used which is an extremely concentrated solution (7–8 M) of cryoprotectants. The most applied PVS is PVS2 solution which contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose in basal MS medium (Matsumoto and Niino 2014, Sakai *et al.* 1990). This solution is supercooled below –70°C and vitrified at about –115°C without any detectable freezing events (Sakai *et al.* 1990, 1991).

Cryopreservation

Preservation of *in vitro* shoot tips and somatic embryos at cryogenic temperatures is considered to be a suitable alternative that can ensure the long-term security of vegetatively maintained germplasm. Once stored in LN, germplasm can be kept for apparently almost unlimited periods, and as a result cryopreservation is the most appropriate for long-term storage of base collections. Cryopreservation is often combined with tissue culture preservation for *in vitro* storage. Because the cryopreservation procedure is usually proceeded by tissue culture, except when preserving seeds, pollen and dormant buds.

In the vitrification method, cells and tissues must be sufficiently dehydrated with plant vitrification solution without causing injury to be capable of vitrifying upon rapid cooling into LN. High survival of *in vitro* grown materials is determined not only by the cryogenic protocol itself, but also by the physiological conditions of the materials to be cryopreserved. This means that some steps for acquisition dehydration tolerance or low temperature tolerance are crucial in the cryopreservation procedure. Following procedures such as preconditioning, preculture and osmoprotection (loading treatment) are vital for successful cryopreservation (Sakai *et al.* 2008). The materials must be in an optimal physiological and morphological state to ensure high recovery and vigorous regrowth after LN exposure (Engelmann *et al.* 2008, Sakai *et al.* 2008). In preconditioning, homogeneous specimens in terms of size, cellular composition, physiological state and growth stage, increase the chances of a positive and uniform response to treatment with loading solution (LS) and PVS2 (Niino 2006). Cold-hardening and preculture of shoot tips with sucrose-enriched media is effective for improving the post-thaw survival of some temperate and tropical species (Takagi *et al.* 1998), due to increased membrane stability (Kaczmarczyk *et al.* 2012). During preculture on sucrose-enriched medium, concentrations of sugar, starch and proline are greatly increased in the shoot tips and may enhance the stability of membranes under conditions of severe dehydration (Matsumoto and Sakai 2003). In addition, a cryoprotective or osmoprotective treatment with LS solution appears promising as a means of enhancing the dehydration tolerance of shoot tips of several species (Matsumoto 2002, Matsumoto and Niino 2014). The protective effect of this solution in cellular peri-protoplasmic spaces may be due to mitigation of the large osmotic stress from exposure to PVS2, as well as to some mechanisms that minimize the injurious membrane changes from severe dehydration (Crowe *et al.* 1988, Steponkus *et al.* 1992).

The optimal dehydration time with PVS2 or air dehydration is also important. An accurate control of the dehydration procedures and prevention of chemical toxicity injuries or excess osmotic stresses during dehydration treatment are indispensable for successful cryopreservation. Optimal dehydration time is species-specific, and may vary with the size, stage and morphological state of the shoot tips (Sakai *et al.* 2008). Oxidative processes involved in cryopreservation protocols may be responsible for the reduced viability of explants after LN exposure. Adding antioxidants in the cryoprotectant or the recovery media that counteract these reactions may improve recovery (Uchendu *et al.* 2010a, 2010b). Polyvinylpyrrolidone (PVP) and plant regulators supplementation in the recovery medium also increase regrowth significantly compared with no supplementation. PVP may be involved in adsorbing the phenolic compounds produced by dead cells (Niino *et al.* 2003).

Practical cryo-storage

During the last 25 years, several cryopreservation techniques have been established based on the conventional slow freezing method. These techniques such as the vitrification method, encapsulation/dehydration method and encapsulation/vitrification method, involve the steps of extraction of freezable water from the tissue cells before cooling

(Reed 2008). As a result, vitrification of internal solutes takes place during cooling. Modified techniques have been developed which further reduce the chance for lethal ice-crystal formation through the application of ultra-fast cooling and rewarming rates. These techniques are called the droplet vitrification method, V cryo-plate method and D cryo-plate method (Niino *et al.* 2013, Panis *et al.* 2005, Yamamoto *et al.* 2011b). Detailed descriptions of these cryopreservation protocols can be found in Reed (2008).

The current status of the main cryo-stored germplasm, apart from potato, is shown (**Table 1**). Seed preservation at super low temperature (by vapor or liquid phase of LN) has been successfully achieved for a wide range of crop species by the standard seed bank protocol. There are several large cryopreserved collections of orthodox seeds. In National Center for Genetic Resources Preservation (NCGRP), USA approximately 10% of the seed accessions preserved (over 37,000 accessions) have been cryopreserved. Whereas, more than 1,200 seed accessions of 50 species have been cryopreserved at the National Bureau of Plant Genetic Resources (NBPGR), India and 400 *Panax ginseng* seed accessions have been cryopreserved in National Agrobiodiversity Center, Rural Development Administration (NAC, RDA), Rep. Korea. At the Institute de Recherche pour le Développement (IRD), France, a cryopreserved collection of coffee germplasm (7 species, over 500 accessions) have been also stored safely in LN even though it is a non-orthodox seed (Engelmann personal communication 2014).

Some temperate woody plants can be cryopreserved by using dormant buds (Towill and Ellis 2008) and this cryopreservation method is called 'Cryopreservation of dormant buds'. This method is now applied to *Malus spp*. (apple, Forsline *et al.* 1998), *Morus spp*. (mulberry, Niino 2000, Rao 2009) and *Ulmus spp*. (elm, Harvengt *et al.* 2004) at four different Institutes having a large scale cryo-storage infrastructure (**Table 1**). Cryopreservation protocol for mulberry dormant buds developed by National Institute of Agrobiological Sciences (NIAS), Japan is as follows. Mulberry branches with axillary buds are collected during the winter season when the buds are quiescent. After harvest, dormant buds with vascular tissue are removed and packed in polypropylene cryotube (8 ml) which are then cooled down to 0°C, –5°C, –10°C, –15°C and –20°C at successive one day intervals as a pre-freezing dehydration process. The final day when buds are at –20°C, the cryotube is removed from the cooling unit and quickly transferred into the vapor phase of LN tank (ca. –160°C) for long-term storage (Niino 2000). The regrowth of 'Kenmochi' mulberry buds stored for 11.5 years in vapor phase of LN tank was 98% by tissue culture after rewarming (Fukui *et al.* 2011).

The large scale cryo-storage of *in vitro* shoot tips has been accomplished at several Institutes by optimizing cryopreservation protocols (**Table 1**). The International Network for the Improvement of Banana and Plantain (INIBAP) has been maintaining the *Musa spp.* cryo-bank collection of over 700 accessions by the droplet vitrification method (Panis *et al.* 2005, Panis 2008). The other crops which have been cryopreserved in cryo-banks, are *Pyrus spp*. (pear, Reed 1990), *Rubus spp*. (raspberry, Reed 1988), *Manihot esculenta* (cassava, Escobar *et al.* 1997), *Allium sativum*

Table 1. Current status of main cryo-storage in the world except potato germplasm

Institute	Materials	Plants	Cryo-storage accessions (No.)	Cryopreservation methods
NCGRP, USA	Orthodox seeds	10% seeds of accession	over 37,000	Desiccation, (Engelmann personal communication 2014)
NBPGR, India	Orthodox seeds	50 species	1,200	
NAB, RDA, Rep. Korea	Orthodox seeds	*Panax ginseng*	400	
IRD, France	Non orthodox seeds	*Coffea spp.*	500	
NCGRP, USA	Dormant buds	*Malus spp.*	2,200	Cryopreservation using dormant buds (Slow freezing)
NIAS, Japan	Dormant buds	*Morus spp.*	1,236	
NBPGR, India	Dormant buds	*Morus spp.*	329	
AFOCEL, France	Dormant buds	*Ulmus spp.*	440	
NCGRP/NCGR, USA	In vitro shoot tips	*Pyrus spp.*	100	Slow freezing
NCGRP/NCG, USA	In vitro shoot tips	*Rubus spp. et al.*	57	Slow freezing
CIAT, Colombia	In vitro shoot tips	*Manihot esculenta*	480	Droplet vitrification
INIBAP, Belgium	In vitro shoot tips	Musa spp.	700	Droplet vitrification
NICS, RDA, Rep. Korea	Shoot from cloves	*Allium sativum L.*	300	Droplet vitrification
IPK, Germany	In vitro shoot tips	*Allium sativum L.*	101	Vitrification
IPK, Germany	In vitro shoot tips	*Mentha* L.	86	Droplet vitrification
SARC, Japan	In vitro shoot tips	*Wasabia japonica* M.	40	Vitrification
NIAS, Japan	In vitro shoot tips	*Juncus effusus*	50	D cryo-plate

These information obtained in 2nd International Symposium on Plant Cryopreservation (Aug. 2013), Fort Collins, Colorado, USA, except seeds. NCGRP (National Center for Genetic Resources Preservation); NCGR (National Clonal Germplasm Repository); NBPGR (National Bureau of Plant Genetic Resources); NAC, RDA (National Agrobiodiversity Center, Rural Development Administration); IRD (Institute de Recherche pour le Développement); NCGR (National Clonal Germplasm Repository); NIAS (National Institute of Agrobiological Sciences); AFOCEL (Association Forêt Cellulose); CIAT (International Center for Tropical Agriculture); INIBAP (International Network for the Improvement of Banana and Plantain); NICS RDA (National Institute of Crop Science, Rural Development Administration); IPK (Leibniz Institute of Plant Genetics and Crop Plant Research); SARC (Shimane Agriculture Research Center).

Table 2. Current cryo-storage status of *in vitro* grown shoot tips of potato

Institute, country	Total accessions	Number of accessions				Cryopreservation methods	Literature
		Field preservation	Seed storage	*In vitro* storage	Cryo-storage		
IPK/GLKS, Germany	6,124 (2,846)	89	2,846 (2,846)	2,855	1,436	DMSO droplet vitrification	Keller personal communication 2014
CIP, Peru	6,768 (2,414)	3,931	6,125 (2,289)	4,062 (49)	869	Droplet vitrification & Vitrification	Ellis and Panta personal communication 2014
Northern Region 6, USA	5,808						Jenderek personal communication 2014
NCGRP, USA					247	Droplet vitrification	
NICS, RDA, Rep. Korea	1,223	670		1,223			Kim personal communication 2014
NAC, RDA, Rep. Korea					130	Droplet vitrification	
NIAS, Japan	1,964	1,964		20	20	V cryo-plate	Yamamoto (2013)
NCSS, Japan				130			
KAES HRO, Japan	500	500				Encapsulation vitrification	Hirai (2011)
CAES HRO, Japan					100		

( ) means number of wild potato accessions.

IPK (Leibniz Institute of Plant Genetics and Crop Plant Research); GLKS (The Groß Lüsewitz Potato Collection); CIP (International Potato Center); NR6 (The US Potato Center); NCGRP (National Center for Genetic Resources Preservation); NICS RDA (National Institute of Crop Science, Rural Development Administration); NAC, RDA (National Agrobiodiversity Center, Rural Development Administration); NIAS (National Institute of Agrobiological Sciences); NCSS ((National Center of Seeds and Seedlings); KAES HRO (Kitami Agricultural Experiment Station, Hokkaido Research Organization); CAES HRO (Central Agricultural Experiment Station, Hokkaido Research Organization).

(garlic, Keller 2005, Kim *et al.* 2004a, 2004b), *Menta L.* (mint, Senula *et al.* 2007), *Wasabia japonica* (wasabi, Matsumoto *et al.* 1994, 1998), *Juncus effuses* (mat rush, Niino *et al.* 2013) (**Table 1**).

Practical cryo-banking of *in vitro* grown potato shoot tips

The current status of potato cryo-banks of *in vitro* grown shoot tips globally is shown (**Table 2**). IPK, Germany, and CIP, Peru, are two of the largest potato genebanks. Both institutes have been applying cryo-storage to potato and achieved large cryo-bank collections with over 1,456 and 869 accessions, respectively. The cryo-storage of the potato shoot tips have been also established at NCGRP, USA, NAC RDA, Rep. Korea, Central Agricultural Experiment Station, Hokkaido Research Organization (CAES HRO), Japan, and NIAS, Japan (**Table 2**). The cryopreservation methods used in these cryo-banks are DMSO droplet (IPK), droplet vitrification (CIP, NCGRP and NAC RDA), encapsulation vitrification (CAES HRO) and V cryo-plate (NIAS). With these methods, except encapsulation vitrification method, shoot tips are directly immersed in LN and in the rewarming solution on aluminum foil strips or cryo-plates. Cooling and warming rates are about 4,000–5,000°C/min and about 3,000–4,000°C/min, respectively, resulting in little or no crystallization and high regrowth after rewarming (Niino *et al.* 2013). In contrast, conventional methods such as vitrification and encapsulation vitrification use capped cryotubes for immersion into LN and retrieval from LN. When using capped cryotubes, cooling and warming rates are about 100–200°C/min and about 80–120°C/min, respectively, which are far less than new methods and this has a great impact on regrowth of cryopreserved materials (Niino *et al.* 2013).

DMSO droplet in IPK

Schäfer-Menuhr *et al.* (1994) developed the ultra-rapid cooling method for *in vitro* shoot tips of potato called the DMSO droplet method, by using aluminium foil. Shoot tips are treated with a 10% DMSO in liquid MS medium with 30 g/l sucrose, 0.5 mg/l zeatin riboside, 0.2 mg/l GA3 and 0.5 mg/l IAA (MSTo, Towill 1983) at room temperature (RT) for 2 h and frozen ultra-rapidly by direct immersion into LN in a 2.5 µl droplet of the same solution on a small piece of aluminium foil (Schäfer-Menuhr 1996). The procedure is shown (**Table 3**) (Kaczmarczyk *et al.* 2009, 2011, Keller *et al.* 2008). This protocol is very simple because only 10% DMSO is used as cryoprotectant solution. The explants (2–3 mm) are incubated in MSTo medium overnight at 22°C and treated with cryoprotectant solution (10% DMSO in MSTo medium) for 1–3 h at RT followed by transfer into droplets of 2.5 µl cryoprotectant solution one by one on aluminium foil. Afterwards the aluminium foil is immersed directly into cryotube filled with LN. The explants are rewarmed quickly by putting aluminium foils in liquid MS medium with 30 g/l sucrose at RT for regeneration. The shoot tips are plated on solid MSTo medium. Currently 1,436 potato accessions are stored at IPK using this protocol with a mean regeneration percentage of 46% (Kaczmarczyk *et al.* 2011). The DMSO droplet method is currently being improved. One improvement is the application of alternating preculture temperature for cold accumulation of shoots under 8-h photoperiod at 21/8°C day/night temperature for 7 days. The other improvement is the adoption of solid medium for regeneration (Kaczmarczyk *et al.* 2008).

Table 3. Five practical cryopreservation methods for *in vitro* grown shoot tips of potato

Procedure	DMSO droplet method in IPK, Germany	Droplet vitrification method in CIP, Peru	Droplet vitrification method in NAC, RDA, Rep. Korea	Encapsulation vitrification method in CAES, HRO, Japan	V cryo–plate method in NIAS, Japan
Culture	Culture on solid Murashige and Skoog medium (MS) with 20 g/l sucrose under 16 h photoperiod at 22°C for 3–4 weeks.	Culture on solid MS with 2 mg/l glycine, nicotinic acid, 0.5 mg/l pyridoxine, 0.1 mg/l thiamine, 25 g/l sucrose and 2.8 g/l phytagel at 22°C, 45 µmol/m^2/s illumination and 16 h light.	Culture nodal segments on solid MS with 30 g/l sucrose, 2.2 g/l phytagel (Sigma Co.) at 24°C, under 16 h light, 100–140 µmol/m^2/s illumination for 3–7 weeks.	Culture nodal segments on solid MS with 0.5 g/l casamino acid, 30 g/l sucrose, 2.5 g/l gellan gum at 23°C and 16 h photoperiod with light intensity of 50 µmol/m^2/s every 2 weeks.	Culture on solid MS with 30 g/l sucrose, 0.3 g/l $CaCl_2$ and 8 g/l agar at 20°C and 16 h photoperiod with light intensity of 104 µmol/m^2/s every 3 months.
Preconditioning	Cold preculture under 8 h photoperiod at 21/8°C day/night temperature for 7 days.	Shoot multiply on the MS using apical cutting (~0.7 cm) for 3 weeks. If need, cold preculture on MS medium with 24 g/l sucrose at 6°C for 7 days.		Culture nodal segments on the MS for 7 days under standard condition or for 1 day under standard condition, then transfer at 4°C and 12 h photoperiod with light intensity of 20 µmol/m^2/s and for 3 weeks.	Culture nodal segments with a lateral bud (about 5 mm) on the solid MS for about 2 weeks in the standard condition.
Shoot tips	Explants of 2–3 mm in length and 0.5–1 mm width.	Explants of 1.8–2.5 mm length apical shoot tips.	Axillary shoot tips (1.0–2.0 mm in size) are isolated by dissection from the upper to middle part of the mother-plants.	Excision shoot tips with 2 to 3 leaflets (about 1 mm length) from axillary buds.	Excision shoot tips with 2 leaflets (about 1.5 mm length) from shoots.
Preculture Mounting shoot tips on cryo-plate/ Encapsulation	Incubate in liquid MS with 30 g/l sucrose, 0.5 mg/l zeatin riboside, 0.2 mg/l GA_3 and 0.5 mg/l IAA (MSTo) overnight at 22°C.	Place specimens on potato meristem medium, which is MS with 0.04 mg/l kinetin, 0.1 mg/l gibberellic acid, 24 g/l sucrose and 2.8 g/l phytagel (MMP) and incubated at RT for about 1 h.	Isolated shoot tips were precultured in liquid MS with 0.3 M sucrose for 8 h under standard conditions. Shoot tips were further precultured in liquid MS with 0.7 M sucrose for 18 h under the same conditions.	Preculture on MS with 0.3 M sucrose, 1 mg/l GA_3, 0.01 mg/l BA and 1 µg/l NAA for 16 hr at 23°C. Encapsulation of shoot tip (one shoot tip/ bead); mixture of shoot tips and calcium-free MS with 2% Na-alginate and 0.4 M sucrose are dropped into 0.1 M $CaCl_2$ soution with 30 g/l sucrose for 30 min, at 25°C.	Preculture on MS with 0.3 M sucrose overnight (16 hr) at 25°C. Pour 2% Na-alginates solution in the wells of cryo-plate. Place the precultured shoot tips one by one in the wells. Then, drop over them 0.1 M $CaCl_2$ soution and wait for 15 min at 25°C for complete polymerization.
Osmoprotection	Treatment with cryoprotectant solution (10%DMSO in MSTo at RT for 1–3 h.	Treatment with osmoprotective solution (LS) with 2 M glycerol and 0.4 M sucrose at RT for 15 min.	Dehydration in 10 ml PVS2 solution for 20 min with continuous shaking (60 rpm).	Treatment beads with MS solution with 0.6 M sucrose, 2 M glycerol and the same plant hormones in preculture medium on rotary shaker (60 rpm) at 25°C for 90 min.	Treatment with LS solution contains 2 M glycerol + 0.8 M sucrose by transfer the cryo-plates in a 25 ml reservoir filled with 20 ml LS at 25°C for 30 min.
Dehydration		PVS2 treatment on ice. Shoot tips were exposed for 50 min to 2 ml ice-cooled PVS2 in glass vials.		Dehydration with PVS2 at 0°C for 3 hr. The beads in PVS2 are shaken (20 rpm) in a water bath.	Dehydration with PVS2 by placing cryo-plates in a 25 ml filled with 20 ml PVS2 at 25°C for 30 min.
Transfer the explants	Transfer the explants into droplets of 2.5 µl cryoprotectant solution one by one on aluminium foils (5 × 25 × 0.03 mm).	The 3 min before the end of each PVS2 treatment, the specimen were transfered to a PVS2 drop (10–15 µl) on an aluminum foil strip (0.5 × 2 cm).	A few min before plunging in LN, seven drops (2.5 µl each) of PVS2 solution are placed on an aluminum foil strip (7 × 20 mm) using a dispenser. One shoot tip is put in each of the seven PVS2 drops.		
Storage	Put the aluminium foils directly into cryotube filled with LN. Close the cryotube refilled with LN and stock into the Dewar with LN.	The strip holding the shoots is then rapidly plunged into a LN filled cryo-tube in a Petri dish on ice. Close the cryotube refilled with LN and stock into LN tanks.	The foil strip is immediately plunged in LN. After a few min, two foil strips is transferred in one 2 ml cryovial, which have been previously filled with LN and stored in a LN tank.	Cryotube containing beads and PVS2, is directly plunged in LN.	Transfer the cryo-plate in 2 ml cryotube, which is held on cryo-cane, and plunge it directly in LN.
Rewarming	Putting aluminium foils quickly in liquid MS with 30 g/l sucrose at room temperature (RT).	Dropping the strips quickly in liquid MS with 1.2 M sucrose at RT and incubate for 20 min.	Foil strips are taken out of the cryovials and immediately plunged in 6 ml 0.8 M sucrose solution at 40°C for 30 s and 6 ml of the solution are added. Shoot tips are further incubated in the unloading solution at RT for 30 min.	Cryotube is put into water bath at 38°C for 3 min. After removing the PVS2 solution, the beads are washed with 1 ml of 1.2 M sucrose solution for 10 min.	Cryo-plate is retrieved from the cryotube in LN and immersed in 2 ml cryotube containing 2 ml MS basal medium with 1 M sucrose, in which it is incubated for 15 min at RT.
Regeneration	Shoot tips are plated on solid MSTo (10 g/l agar) under a 16 h photoperiod at 22°C.	Post-cryo culture in the dark on MMP with progressively decreased sucrose levels (daily transfers from 0.3, to 0.2, to 0.1 M and maintained on 0.07 M). One week after warming, shoot tips were transferred from the filter paper to fresh MMP (0.07 M sucrose) and incubated at 22°C under standard condition.	Shoot tips are post-cultured on semi solid MS with 0.05 mg/l IAA, 0.3 mg/l zeatin, 0.05 mg/l GA_3, 30 g/l sucrose and 1.8 mg/l phytagel at 24°C under low light intensity for 7 days and then transferred to standard culture conditions.	Beads with shoot tips are plated on the solid MS with 30 g/l sucrose and the same plant hormones in preculture medium for 1 day, then transferred on the solid MS with 30 g/l sucrose and 0.5 µg/l GA_3 under standard condition.	Shoot tips are plated on the solid MS and cultured under standard condition.
Reference	Kaczmarczyk *et al.* 2011 Keller *et al.* 2008	Panta *et al.* 2014	Kim *et al.* 2006 Yoon *et al.* 2006	Hirai and Sakai 1999	Yamamoto *et al.* 2013 Yamamoto *et al.* 2011b

Kaczmarczyk *et al.* (2011) emphasized that a critical aspect in potato cryopreservation is the diverse response between different genotypes in terms of their regeneration capacities after cryopreservation. Therefore, genotypes not responsive to cryopreservation still need to be maintained using *in vitro* storage or field storage. In addition, certain genotypes may not even have tissue culture ability and need to be preserved as tubers (Keller *et al.* 2011). Finally, none of the conservation strategies (cryopreservation, *in vitro* storage, field storage) can be completely safe, because materials may always be lost regardless of the conservation technique.

Droplet vitrification in CIP

There are several variations of the vitrification method. The discrimination of these methods is that the standard vitrification protocol takes place in a cryotube while the droplet vitrification protocol is done on aluminium foil strips, and encapsulation vitrification adds an encapsulation step to the standard protocol (Panis 2008). The droplet vitrification method was developed in banana cryopreservation at first resulting increase in a regrowth rate of 40–50% compared to standard vitrification (Panis *et al.* 2005). In the droplet vitrification method, shoot tips are plated on aluminium foil during cooling and dehydrated with highly concentrated vitrification solution such as PVS2. Rewarming after cryopreservation resulted in regrowth of 46–51% (Halmagyi *et al.* 2005), 8–47% (Panta *et al.* 2006) and 64– 94% (Kim *et al.* 2006). These results confirm that regrowth ability of cryopreserved potato shoot tips is genotype-dependent as indicated by Kaczmarczyk *et al.* (2011). Genotype dependency was also observed by Panta *et al.* (2009) as the results showed that higher linoleic acid content in frost resistant genotypes of potato, are positively correlated with higher regeneration rates after cryopreservation.

The procedure of the latest droplet vitrification in CIP are shown (**Table 3**) (Panta *et al.* 2014). The shoot tips (1.3–2.5 mm) are dissected from the shoots preconditioned and incubated at RT for about 1 h on potato meristem medium containing 24 g/l sucrose, 0.04 mg/l kinetin and 0.1 mg/l gibberellic acid (MMP, Panta *et al.* 2014). Osmoprotection is performed by LS (MS medium with 2 M glycerol and 0.4 M sucrose) for 15 min at RT. After that shoot tips are dehydrated by PVS2 for 50 min at 0°C and 3 min before the end of each PVS2 treatment, the shoot tips are transferred to a PVS2 drop (10–15 μl) on an aluminum foil strip (0.5 × 2 cm). Then, the strip holding the shoots are rapidly immersed into a LN filled cryo-tube in a Petri dish on ice. The strips are then rewarmed quickly by dropping them in liquid MS medium with 1.2 M sucrose at RT and incubated for 20 min for regeneration. Post-cryo culture are kept in the dark on MMP with a progressive decrease in sucrose levels (daily transfers from 0.3, to 0.2, to 0.1 M and maintained on medium with 0.07 M sucrose). One week after plating, shoot tips are transferred from the filter paper to fresh MMP (0.07 M sucrose) and incubated at 22°C under standard conditions. This protocol was successfully applied to 4 genotypes showing different reactions to abiotic stress, which had high regrowth levels ranging from 23% to 76% (Panta *et al.* 2014). The response to cryopreservation is strongly genotype and species- specific, limiting the use of the current protocols to large diverse collections such as the CIP's potato collection. To overcome this issue, it is crucial not only to optimize the protocol for different genotypes, but also to make a uniform, healthy and robust shoot tips able to tolerate cryopreservation procedures and the regeneration system. Also, it is important that the method is simple and a suitable protocol for large scale application to potato cryopreservation. In the schematic procedure, the treatment combining 15 min LS at RT, 50 min PVS2 at 0°C, apical shoot tips from 3 week old mother plantlets, explant size (1.8–2.5 mm), cooling and warming on aluminium foil strips, produced positive result in the basic protocol for improving potato PVS2 vitrification (Panta *et al.* 2014).

Droplet vitrification in NAC, RDA

The optimization of the protocol for diverse genotypes is a prerequisite for implementing large scale cryopreservation of potato collections in genebanks. The irregular survival levels observed within different potato species could be related to many factors such as subculture conditions, size of the shoot tips and their location on the plantlet axis, sucrose concentration of the preculture medium, preculture time, dehydration, cooling and warming, and unloading step (Kim *et al.* 2006). The subculture conditions, light intensity, aeration and planting density significantly affected survival of cryopreserved shoot tips. Also, the subculture duration and the position of the shoot tips on the axis of the *in vitro* plantlets have a significant effect on survival of cryopreserved shoot tips (Yoon *et al.* 2006).

The procedure of droplet vitrification in NAC, RDA is shown (**Table 3**) (Kim *et al.* 2006, Yoon *et al.* 2006). The shoot tips (1.0–2.0 mm) are dissected from the subcultured shoots. The optimal duration and size of shoot tips were different by genotypes; 7 weeks and 1.5–2.0 mm for 'Dejima' and 5 weeks, 1.0–1.5 mm for 'STN13' (Yoon *et al.* 2006). The shoot tips are precultured with 0.3 M sucrose for 8 h followed by 0.7 M sucrose for 18 h. The precultured shoot tips are dehydrated with PVS2 for 20 min without osmoprotection treatment. A few min before the end of each PVS2 treatment, the shoot tips are transferred to a PVS2 drop (2.5 μl each) on an aluminum foil strip (7 × 20 mm). Then, the strip holding the shoots are rapidly immersed into LN. For rapid warming, the strips are directly dipped in 6 ml 0.8 M sucrose solution at 40°C for 30 s and 6 ml of the solution are added. Shoot tips are further incubated in the unloading solution at RT for 30 min. Shoot tips are post-cultured on semi solid MS with 0.05 mg/l IAA, 0.3 mg/l zeatin, 0.05 mg/l GA_3, 30 g/l sucrose and 1.8 mg/l phytagel at 24°C under low light intensity for 7 days and then transferred to standard culture conditions. This optimized protocol was successfully applied to 12 potato accessions, including wild species, resulting 64–94% regrowth (Kim *et al.*

2006). The same protocol was applied to the Korean *in vitro* potato collection at NAC RDA for cryo-storage of 130 accessions.

The NCGRP USA has also adopted this protocol with slight modifications for potato cryo-storage of 247 accessions. This protocol is at the following Web site; (http://pdf.server4.org/s/solanum-tuberosum-at-ncgrp---usda-w12260.html).

Encapsulation Vitrification in CAES HRO Japan

Originally, the encapsulation-dehydration technique was developed for easy handling of a large number of meristems at the same time using the vitrification method (Matsumoto *et al.* 1995). Hirai and Sakai (1999) applied this method (**Table 3**) to various potato cultivars. In this method, uniform shoot tips from axillary shoots with 2 to 3 leaflets (approximately 1 mm in size) are used which are induced from nodal segments at 23°C for 7 days or at 4°C for 3 weeks. Shoot tips are precultured on MS medium supplemented with 0.3 M sucrose, 1 mg/l GA_3, 0.01 mg/l BA and 1 µg/l NAA overnight at 23°C. After that shoot tips are trapped within alginate gel beads (one shoot tip / one bead, diameter: 4–5 mm) with 0.4 M sucrose. Encapsulated shoot tips are treated with a mixture of 2 M glycerol and 0.6 M sucrose on a rotary shaker (60 rpm) at 25°C for 90 min to induce dehydration tolerance. They are then dehydrated with PVS2 at 0°C for 3 h in a water bath with shaking (20 rpm). The 10–15 beads are suspended in 1 ml PVS2 solution per cryotube and plunged directly into LN. For regeneration, cryotube are put into a water bath at 38°C for 3 min. After removing the PVS2 solution, rewarmed beads are washed with 1 ml of 1.2 M sucrose solution for 10 min. Then, beads with shoot tips are plated on the solid MS medium having 3% sucrose and the same plant hormones as in preculture medium for 1 day, then transferred on the solid MS medium with 30 g/l sucrose and 0.5 µg/l GA_3 under standard condition. This protocol was successfully applied to 14 potato accessions resulting in 47–71% regrowth (Hirai and Sakai 1999). The protocol was applied to Hokkaido Research Organization (HRO) potato collection and in CAES HRO, 100 accessions have been cryopreserved in LN tank by using this method (Hirai 2011).

V cryo–plate in NIAS

In conventional vitrification procedures, including droplet vitrification, small size shoot tips are suspended in various solutions employed, which have to be removed and added by repeated pipetting. This often results in damage and loss of shoot tips during the course of the cryopreservation protocol. Moreover, vitrification procedures require a precise control of duration of treatment with vitrification solutions due to the narrow range of optimal treatment durations. In the droplet vitrification procedure, dehydrated shoot tips also need to be transferred on aluminium strips just before immersion in LN (Yamamoto *et al.* 2011b). The vitrification method using aluminium plates, named V cryo-plate method was developed in order to establish a simple, reproducible and reliable protocol using aluminium cryo-plates. This new method is now adopted for several plant species such as strawberry (Yamamoto *et al.* 2011a), Dalmatian chrysanthemum (Yamamoto *et al.* 2011b), mint (Yamamoto *et al.* 2012b), mulberry (Yamamoto *et al.* 2012a), carnation (Sekizawa *et al.* 2011), mat rush (Niino *et al.* 2013) and sugarcane (Rafique *et al.* 2014). It is a user-friendly procedure and permits high cooling and warming rates of treated materials. As a result, superior regrowth is obtained after cryopreservation for the plant species which have been tested by this method.

Currently, the V cryo-plate method was successfully applied to *in vitro* grown potato (Yamamoto *et al.* 2013). The procedure is shown in **Table 3**. The shoots from nodal segments are cultured on solid MS medium containing 30 g/l sucrose and 0.3 g/l $CaCl_2$ at 20°C for 2 weeks. The shoot tips (about 1.5 mm) are excised from the *in vitro* grown shoots and precultured on MS medium containing 0.3 M sucrose at 25°C for overnight. The precultured shoot tips are placed on aluminium cryo-plates (7 mm × 37 mm × 0.5 mm) with ten wells (diameter 1.5 mm, depth 0.75 mm) and embedded with calcium alginate gel. Osmoprotection is performed by immersing the cryo-plates for 30 min at 25°C in 25 ml pipetting reservoirs filled with LS (MS medium with 2 M glycerol and 0.8 M sucrose). For dehydration, the cryo-plates are transferred and immersed in another reservoirs filled with PVS2 for 30 min at 25°C. Then, the cryo-plate is transferred in an uncapped 2 ml cryotube and directly plunged into LN. For rewarming, the cryo-plate is retrieved from the cryotube in LN and immersed in a 2 ml cryotube containing 2 ml MS basal medium with 1 M sucrose, in which it is incubated for 15 min at RT. Rewarmed shoot tips are placed on solid MS medium and cultured under standard conditions. This protocol was successfully applied to 16 cultivars and 4 wild potato accessions, resulting in high regrowth levels ranging from 93% to 100%. In genebank NIAS Japan, 1,964 accessions of potato GRs are currently maintained in the field. Now, cryopreservation is a preservation option of potato GRs, as a long-term back-up system for the field collections. Currently, an air-dehydration method is being applied using aluminium cryo-plates named D cryo-plate method, which combines the encapsulation-dehydration method and V cryo-plate method (Niino *et al.* 2013). In this method, shoot tips/buds attached to the cryo-plates are dehydrated under the laminar air flow cabinet's air current after treating with LS. Air dehydration can minimize damage to specimens by avoiding the use of PVS2, using materials with comparatively higher moisture content (MC) and performing minimal excision of young leaves and/or sheaths. In *in vitro* mat rush buds, the D cryo-plate method overcame problems associated with sensitivity to PVS2, insufficient or excessive dehydration, damage to and loss of material during excision and manipulations (Niino *et al.* 2013, 2014). This method is going to be applied for *in vitro* grown shoots of potatoes.

Longevity and Genetic Stability

Germplasm will survive for a long time in cryogenic storage, it is not known for exactly how long. Estimates of the actual shelf life of cryogenically stored material are critical for efficient gene-banking, but are difficult to obtain because of instrument limitations or the extended times required for measurements. The seeds of *Brassica oleracea* cryo-stored in LN for 20 years maintained their viability up to 97% after storage, but the seeds stored at –18°C for 25 years had lower viability (11%) (Walters *et al.* 2004). Cryogenic storage clearly prolonged shelf life of lettuce seeds with half-lives projected as ~450 and ~2600 years for fresh lettuce seeds stored in the vapor and liquid phases of liquid nitrogen, respectively (Walters *et al.* 2004).

Maintaining viability and genetic stability during storage is also important for cryopreserved *in vitro* shoot tips. After the development of vitrification methods, a few research publications appeared to suggest the exact viability and genetic stability of materials after long-term cryo-storage. Recently, Caswell and Kartha (2009) demonstrated that it was possible to cryopreserve *in vitro* strawberry and pea meristems in LN for 28 years. In the case of *in vitro* grown strawberry meristems, there was no decrease in the percentage of viable meristems stored for 8 weeks or 28 years. This result significantly extends the reported duration of successful cryopreservation of plant meristems and provides corroborating evidence to the theory that plant meristems may be cryopreserved indefinitely (Caswell and Kartha 2009). Also, for *in vitro* grown wasabi shoots, there was no significant differences of regrowth and morphological characteristics among 10 year cryo-storage, 2 h cryo-storage, treated control and control by the vitrification method (Matsumoto *et al.* 2013). In biochemical analyses of sinigrin, which is a chemical precursor of the mustard oil, there was no significant difference of concentration level among them. All restriction fragment length polymorphism (RAPD) fragment patterns of 10 year cryo-storage tested were identical to those of 2 h cryo-storage. From these results, Matsumoto *et al.* (2013) concluded that wasabi plants derived from shoot tips cryopreserved for 10 years by vitrification method are genetically stable. Charoensub *et al.* (2004) suggested that callus formation might increase the frequency of genetic variants. However, optimized cryogenic techniques with suitable conditions can provide high survival after rewarming. High survival is attributed to a lower degree of injury incurred by explants during cooling and rewarming. This indicates that a high level of survival after cryopreservation is necessary to reducing genetic changes.

In potato, many studies on genetic integrity after cryopreservation have been reported. Morphological and phenotypic, cytological and molecular comparisons were conducted revealing that plant material was genetically stable as a result of cryopreservation (Kaczmarczyk *et al.* 2011). Potato shoot tips cryopreserved by the DMSO droplet method and stored in LN for several years was found to have no adverse effect on the regeneration rates (Mix-Wagner *et al.* 2003). The genetic stability was also confirmed using morphological parameters, flow cytometric measurements and RFLP analyses, concluding that the cryopreservation technique may not induce somaclonal variation (Schäfer-Menuhr *et al.* 1997).

To date, many molecular and morphological study approaches for the genetic stability of cryopreserved plants had been reported. No observable significant differences have been observed in the regenerated material (Matsumoto *et al.* 2013). Using a biochemical approach, the diosgenin content in *Dioscorea deltoidea* (Dixit-Sharma *et al.* 2005) and the ginsenoside content in *Panax ginseng* (Yoshimatsu *et al.* 1996) were analyzed and found to be the same as those of controls. However, the possibility of some genetic changes may occur in cryopreserved plants (Kaity *et al.* 2008, Martin and Gonzalez-Benito 2005, Peredo *et al.* 2008). It is thus necessary to monitor the genetic stability of regenerated plants (true-to-type status) as a result of cryopreservation.

Conclusion

Over the last 25 years, several countries have been conserving PGR by cryopreservation. The cryopreservation technique is an effective approach for storage of plant cells, tissues, seeds and embryos. The development of new techniques for cryopreservation such as droplet vitrification, DMSO droplet and V cryo-plate methods, is facilitating the systematic and strategic cryobanking of PGRs. Cryostorage of potato germplasm is at the cutting-edge of cryopreservation research. Many experiences obtained from potato cryo-banking in IPK, CIP, NAC RDA, CAES HRO and NIAS provide insights into the storage, principles and pitfalls governing the operations of a cryo-bank. These new techniques have facilitated the cryo-banking of other plant species that are established tissue culture techniques like *Musa* germplasm. Reed (2008) summarized practical issues that need to be resolved before initiation of a cryo-bank. These issues include the plant materials to be preserved, storage records, storage forms, quantity to store, protocol testing, storage controls and recovery, as well as facilities and equipment. In the case of potato where huge diversity exists the genotype needs to be considered in the routine protocol of cryopreservation.

It is important to have many choices of protocol for cryopreservation, because there are many types of plant propagules and plant species to be cryopreserved. The first thing that should be done is to determine how to make materials for cryopreservation, which are not only healthy and vigorous shoots, but also at a uniform stage and size. Despite research conducted to date some genotypes remain ‘recalcitrant materials’. Secondly, it is necessary to determine whether the specimens are sensitive to some chemicals such as PVS or excess dehydration. Thirdly, the unloading step and regeneration medium should be reexamined. Current new protocols apply rapid cooling and warming by direct immersion in LN and unloading solution. Several papers report that optimal exposure time to PVS2 shows a wide spectrum of efficiency (Sekizawa *et al.* 2011, Tanaka *et al.* 2011, Yamamoto *et al.* 2012a, 2012b). This means high regrowth might be obtained by rapid cooling and warming, even though the water content of specimen has not reached optimal.

Cryopreservation should be considered as a backup to field collections to insure against loss of plant germplasm (Niino *et al.* 2007). Priority of collections to be cryopreserved should be given to the 'at risk' plants that have an increased chance of being lost from a collection. Some minor and endangered crops have genotypes that are difficult to establish in *in vitro* culture. It is crucial for successful cryopreservation to develop efficient micro propagation systems. Developing tissue culture systems which allow rapid multiplication and which stimulate regrowth after retrieving samples from LN will be the next challenge for cryopreservation research.

In this review, we show practical protocols which are used in potato cryo-banks have been described. These protocols are useful techniques for cryopreservation of difficult to conserve potato genotypes, wild type potatoes, and other plant species after marginal modifications. Also, the protocols might efficiently complement one another. For example, the D cryo-plate method combines encapsulation dehydration with the V cryo-plate method. The physical dehydration employed in the D cryo-plate protocol might be more uniform, thus explaining the high regrowth obtained with larger shoot tips cryopreserved with the D cryo-plate (Niino *et al.* 2013, 2014). The D cryo-plate method may be used with larger explants, which are very sensitive or less sensitive to physical damage and cryoprotectant toxicity. To realize comprehensive cryo-storage of PGR further development of cryopreservation techniques developed are required.

Acknowledgements

We are grateful to Dr. T. Rafique for critical reading of the manuscript and to Dr. F. Engelmann for suggestions. Also, we are thankful to Drs. E.R.J. Keller, H-H. Kim, M. Jenderek, A. Panta and D. Ellis for their information related to cryopreservation. This work was supported by JST/JICA, SATREPS: Diversity Assessment and development of Sustainable Use of Mexican Genetic Resources.

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