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Cryopreservation of Potato Shoot Tips

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ABSTRACT

Potato is one of the most important staple crops in both developed and developing countries of the world. Potato genetic resources are a prerequisite for potato breeding in both conventional and genetic engineering programmes. Cryopreservation has long been considered an ideal means for the long-term conservation of plant genetic resources. This review provides comprehensive information on variously novel cryogenic techniques that have been developed since 1990. Factors affecting the success of cryopreservation of potato shoot tips are analyzed and discussed. Evidence of morphological and molecular analysis demonstrates that plants regenerated from cryopreserved shoot tips are genetically stable. Efficient elimination of potato viruses by cryotherapy of shoot tips provides an alternative method for virus elimination. Thus, the materials can be simultaneously prepared for virus elimination and long-term preservation. Routine application of cryopreservation of shoot tips for the long-term preservation of potato genetic resources has already started in Germany, Peru, Czech Republic and Korea. Species- and cultivar-specificity has to be taken into account for much wider application when cryo-protocols are to be developed. Fundamental studies on mechanisms by which potato shoot tips withstand freezing in liquid nitrogen would help solve this problem.

Keywords: genetic resources, long-term preservation, shoot tips, virus, *Solanum*
Abbreviations: BAP, 6-benzyl aminopurine; **DMSO**, dimethyl sulfoxide; **GA_3**, gibberellic acid; **IAA**, indole-3-acetic acid; **LN**, liquid nitrogen; **MS**, Murashige and Skoog (1962); **NAA**, naphthalene acetic acid; **PLRV**, *Potato leafroll virus*; **PVS2**, plant vitrification solution 2 (Sakai *et al*. 1990); **PVY**, *Potato virus Y*; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism

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INTRODUCTION

Potato, a member of the Solanaceae family, is native to the Andes Mountains of Peru and Bolivia. The cultivated potato (*Solanum tuberosum* L.) has at least 2400 years of history in cultivation. Potato is globally the fourth largest staple crop next to rice, wheat and maize. During the last two decades, although potato production in developed countries decreased, an increase in potato production throughout the world has continued, mainly due to an increase in developing countries (FOA 2006). In 2006, the total area and yield of the world's potato production reached 19.3 million hectares and 325 million tons, respectively, with the average yield being 16.8 tons per hectare (FOA 2007). China is now the biggest potato-producing country, accounting for more than 20% of the global potato-growing areas and potato yield. Plant genetic resources, including cultivated varieties and wild species, are a prerequisite for potato breeding in both con-

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ventional and genetic engineering programmes (Bradshaw *et al*. 2006). Germplasm conservation has an important role to play in the maintenance of biodiversity and in avoidance of genetic erosion.

Cryopreservation, i.e. storage of biological specimens at the ultra-low temperature of liquid nitrogen (LN, -196°C), is considered as an ideal means for the long-term conservation of plant germplasm (Engelmann 1997). At this temperature, all cellular divisions and metabolic processes essentially cease, and theoretically, plant materials can thus be stored without any change for an indefinite period of time (Engelmann 1997). Moreover, such storage requires a small volume, demands very limited maintenance, and thus lowers the cost. For long-term storage of genetic resources, organized tissues such as shoot tips are preferred over callus and cell cultures because they are genetically more stable (Bajaj 1991).

Potato is one of the most amenable of major crop plants to cryopreservation (Henshaw *et al*. 1985; Mix-Wagner 1999; Gonzalez-Arnao *et al*. 2008). Cryogenic studies on potato started from the late 1970s with the development of ultra-rapid freezing (Bajaj 1977, 1978; Grout and Henshaw 1978; Bajaj 1981; Towill 1981a; Benson *et al*. 1989) and two-step freezing methods (Grout and Henshaw 1978; Towill 1981a, 1981b, 1983, 1984; Benson *et al*. 1989). These so-called conventional methods have disadvantages including low survival, lag phase of recovery and callus formation (Towill 1983, 1984; Henshaw *et al*. 1985; Benson *et al*. 1989). In addition, the two-step freezing method is complicated and time-consuming, and requires a programmable freezer which could be too expensive for many laboratories to use. The conventional methods for cryostorage of potato shoot tips have been extensively reviewed by Henshaw *et al.* (1985), Mix-Wagner (1999) and Gonzalez-Arnao *et al.* (2008). For the long-term storage of genetic resources, development of reliable protocols that produce high recovery and are cost-effective is the basic requirement. Since the 1990s, great efforts have been made to establish novel cryogenic procedures that allow shoot tips to be directly immersed in LN, thus avoiding usage of programmable freezer, and at the same time producing high percentages of survival and plant regeneration of cryopreserved shoot tips. To date, various novel cryogenic protocols have been developed for potato, including droplet freezing (Schäfer-Menuhr *et al*. 1994, 1996, 1997), encapsulation-dehydration (Fabre and Dereuddre 1990; Benson *et al*. 1996; Bouafia *et al*. 1996), vitrification (Towill 1990; Schnabel-Preikstas *et al*. 1992; Sarkar and Naik 1998), encapsulation-vitrification (Hirai and Sakai 1999) and droplet-vitrification (Halmagyi *et al*. 2005; Kim *et al*. 2006; Yoon *et al*. 2006). Successful establishment of these novel cryogenic protocols has made cryopreservation of potato shoot tips most advanced in comparison with other crop species. Now, cryostorage has been routinely used for the long-term storage of potato germplasm in the Institute of Plant Genetics and Crop Plant Research in Germany (Keller and Dreiling 2003; Keller *et al*. 2006), at the International Potato Centre (CIP) in Peru (Golmirzaie and Panta 2000; Panta *et al*. 2006; Gonzalez-Arnao *et al*. 2008), in the Research Institute of Crop Production in Czech Republic (Faltus *et al*. 2006) and at the National Institute of Agricultural Biotechnology in Korea (Kim *et al*. 2006; Yoon *et al*. 2006, 2007). The development and routine application of cryopreservation techniques for tropical crops including potato have been reviewed recently by Gonzalez-Arnao *et al* (2008).

NOVEL CRYOGENIC TECHNIQUES

Droplet freezing

The droplet method, originally described by Kartha *et al.* (1982) for cassava (*Manihot esculenta* Crantz) shoot tips, was successfully modified by Schäfer-Menuhr *et al.* (1994, 1996, 1997) for cryopreservation of potato (*S. tuberosum*) shoot tips. In this procedure, *in vitro* stock plantlets were grown in 12 cm high jars containing Murashige and Skoog (MS, 1962) medium, and maintained at 23°C under light conditions. When the plantlets reached 10 cm in height, shoot tips in 2-3 mm long and 0.5-1 mm thick were excised and incubated at 23°C on MSTo medium. MSTo medium is composed of MS supplemented with 30 g/l sucrose and plant growth regulators described by Towill (1983): 0.5 mg/l zeatin riboside, 0.2 mg/l gibberellic acid (GA_3) and 0.5 mg/l indole-3-acetic acid (IAA). Following incubation, shoot tips were transferred into a cryoprotectant solution made of MSTo containing 10% dimethyl sulfoxide (DMSO). After incubation for 2 h at room temperature, 2.5 µl droplets of the cryoprotectant were pipetted onto heat-sterilized pieces (0.7 × 2 cm) of 0.3 mm thick aluminum foils (6 droplets per foil). One shoot tip was transferred into each droplet and then two foils were transferred into a 2 ml pre-cooled cryotube prior to direct immersion in LN for cryostorage. After thawing at room temperature, shoot tips were post-cultured on MSTo medium for recovery. Regenerated shoots were transferred onto MS medium without any hormones for whole plant regeneration and further micropropagation.

Using 219 varieties and genotypes of potato, Schäfer-Menuhr *et al*. (1997) found that all of them could withstand freezing in LN using the droplet method with an average survival and regeneration percentage reaching 80 and 40%, respectively, for most of the varieties and genotypes tested. The droplet method has been routinely used for the long-term preservation of potato germplasm at the Institute of Crop and Grassland Science in Baunschweig (Schäfer-Menuhr *et al*. 1997; Mix-Wagner *et al*. 2003) and at the Institute of Plant Genetics and Crop Plant Research in Gatersleben of Germany (Keller and Dreiling 2003; Keller *et al*. 2006). Since the year 2002, all the potato cryopreservation was unified and moved to Gatersleben in which 1028 accessions of potato germplasm have to date been cryopreserved (Keller *et al*. 2006).

The droplet method is simple, easy to implement and successful with a large number of potato species and genotypes (Schäfer-Menuhr *et al*. 1996, 1997; Mix-Wagner 1999, 2003; Keller and Dreiling 2003). The procedure is relatively cheap (Schäfer-Menuhr *et al*. 1996, 1997). However, cultivar-specificity is still a major problem which in some cases resulted in low survival (<20%) and low plant regeneration (<40%) (Schäfer-Menuhr *et al*. 1996, 1997). Improvement of survival and regeneration of cryostored shoot tips of some difficult cultivars still needs further studies (Schäfer-Menuhr *et al*. 1996, 1997; Mix-Wagner 1999; Keller and Dreiling 2003).

Encapsulation-dehydration

Using *Solanum phureja*, a diploid species of potato, Fabre and Dereuddre (1990) were the first to develop encapsulation-dehydration for cryopreservation of potato shoot tips. *In vitro* stock shoots were maintained on medium A composed of MS (Murashige and Skoog 1962) minerals, Morel and Wetmore (1951) vitamins, 30 g/l sucrose and 8 g/l agar (pH 5.8). The stock shoots used for cryopreservation were cultured at 20°C, under a light intensity of 50 µE $m^{-2}s^{-1}$ and 70% relative humidity. Apical sections from 8-10 week-old stock shoots were transferred onto medium A for 7 days. Shoot tips of 0.5 mm in size with 3-4 leaf primordia were excised, and collected on medium B consisted of Morel and Muller (1964) macroelements, Heller (1953) microelements, Morel and Wetmore (1951) vitamins, 0.1 M sucrose, 0.001 mg/l NAA, 0.01 mg/l BAP, 5.0 mg/l GA_3 and agar 8g/l (pH 5.8). The shoot tips were suspended in liquid calcium-free medium B supplemented with 3% (w/v) low viscosity sodium alginate. The mixture was dropped into liquid medium B containing 0.1 M calcium chlorite to form beads, each bead being about 3 mm in diameter and containing 1-2 shoot tips. The beads were pre-cultured for 72 h in Erlenmeyer flasks containing liquid medium B supplemented with 0.75 M sucrose. Precultured shoot tips were dehydrated by air drying in a laminar flow at room temperature for

4 h after which the water content of the beads reached about 44% (fresh weight basis). Dehydrated beads were subjected to either direct freezing or two-step freezing. Following slow warming at room temperature, frozen shoot tips were post-cultured on solid medium B for survival and shoot regeneration. Although 19.6% shoot tips withstood direct freezing, none of them developed shoots. With two-step freezing, about 70 and 27% of cryopreserved shoot tips survived and regenerated into shoots.

However, when this encapsulation-dehydration protocol was applied to other genotypes of *S. phureja* and *S. tuberosum* (tetraploidy), survival was strongly genotype- and species-specific (Bouafia *et al*. 1996). Therefore, a modified protocol was proposed (Bouafia *et al*. 1996) in which excised shoot tips were reactivated for 14 days by culturing them on the same medium as used for maintenance of the stock shoots. Following encapsulation, the shoot tips were either stepwise or directly precultured on sucrose-rich medium. Stepwise preculture was performed by culturing the shoot tips on solid medium with progressively increasing sucrose concentrations (0.3, 0.5, 0.75 and 1 M) for 12 h for each concentration, followed by maintaining in 1 M sucrose for 3 days. Direct preculture was carried out by placing encapsulated shoot tips on solid medium containing 0.75 M sucrose for 2 days. Using this modified protocol, all cultivars tested (3 cultivars of *S. phureja* and 2 cultivars of *S. tuberosum*) survived and regenerated into shoots. Average survival of cryopreserved shoot tips of these 5 cultivars was over 65% using direct preculture with 0.75 M sucrose for 2 days. In comparison, overall means of survival were higher in diploid cultivars (70%) than in tetraploid cultivars (57%). These results suggest that diploid cultivars (*S. phureja*) were more tolerant to dehydration than tetraploid cultivars (*S. tuberosum*). When using encapsulation-dehydration for cryopreserving six potato species representing three series and three ploidy levels (diploid, tetraploid and hexaploid), Benson *et al*. (1996) found that although considerable variations in recovery existed between the experimental replicates, and between early (1-2 weeks after thawing) and longer-term (5-6 weeks after thawing) recovery, all potato genotypes studied could survive and regenerate plantlets, with lowest (14%) and highest (32%) plant regeneration obtained for *S. guerreroense* (hexaploid) and *S. acaule* (tetraploid), respectively.

Vitrification

Towill (1990) and Schnabel-Preikstas *et al.* (1992) were the first to successfully apply vitrification for cryopreservation of potato shoot tips. Sarkar and Nail (1998) described in detail the vitrification protocol and applied the established method to five tetraploid potato cultivars (*S. tuberosum*). In this protocol, *in vitro* stock plantlets were maintained on a micropropagation medium composed of MS (Murashige and Skoog 1962) medium supplemented with 8.39 µM D-calcium pantothenate, 0.29 µM GA_3, 0.054 µM naphthalene acetic acid (NAA), 30 g/l sucrose and 8 g/l agar (Sarkar *et al*. 1997). Apical shoot tips of 0.5-0.7 mm long excised from 30-day old plantlets were precultured on filter paper over half-strength liquid MS medium supplemented with 8.7 µM GA_3, 0.3-0.5 M sucrose and 0.2 M mannitol for 2 days under a 16 h photoperiod with a light intensity of 40 µmol $m^{-2}s^{-1}$ at 24°C. Precultured shoot tips were loaded successively with 20% and 60% plant vitrification solution 2 (PVS2) (Sakai *et al*. 1990), with the former for 30 min at room temperature and the latter for 15 min on an ice bath. PVS2 solution contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS medium. Loaded shoot tips were dehydrated with PVS2 at 0°C for 5 min. Following PVS2 treatment, shoot tips were transferred into cryotubes, each containing 5 shoot tips in 0.7 ml PVS2. Cryotubes were then directly immersed in LN for cryostorage. Frozen shoot tips were warmed for 1 min at 35°C and washed with a dilution medium [1.2 M sucrose in liquid MS (Murashige and Skoog 1962)] for 30 min, followed by growing on MS (Murashige and Skoog 1962) medium containing 0.2 M sucrose, 5.8 µM GA_3, 1.0 µM BAP and 6 g/l agar. The cultures were placed under a 16 h diffuse light (6 µmol$m^{-2}s^{-1}$) photoperiod at 24°C for 1 week and then transferred onto MS (Murashige and Skoog 1962) medium supplemented with 0.09 M sucrose, 2.9 µM GA_3 and 6 g/l agar under the normal light conditions for recovery. With the optimized parameters, about 54% of cryopreserved shoot tips survived, of which about 50% directly regenerated into shoots without callus formation.

Kryszczuk *et al.* (2006) compared the effects of the vitrification and droplet method on cryopreservation of potato (*S. tuberosum*) shoot tips and found that average survival and shoot regeneration of cryopreserved shoot tips of four cultivars were significantly higher using the vitrification method (79.8% and 58%, respectively) than the droplet method (36.7% and 13.8%, respectively). Compared with encapsulation-dehydration and encapsulation-vitrification, vitrification is simple, time-saving and also low in cost (Sarkar and Naik 1998). Like other cryogenic procedures, vitrification is also genotype-dependent (Sarkar and Naik 1998; Kryszczuk *et al*. 2006). Up to 2006, the vitrification method has been used for long-term preservation of 446 accessions of potato genetic resources at the International Potato Centre in Peru (Gonzalez-Arnao 2008).

Encapsulation-vitrification

Hirai and Sakai (1999) described an encapsulation-vitrification protocol for successful cryopreservation of potato (*S. tuberosum*) shoot tips. Nodal segments of 3-4 nodes with an apical bud taken from *in vitro* stock shoots were cultured on a basic medium composed of MS (Murashige and Skoog 1962) medium supplemented with 0.5 g/l casamino acid, 30 g/l sucrose and 2.5 g/l gellan-gum, and placed at 23°C under a 16 h photoperiod at 96 mol $m^{-2}s^{-1}$. Following incubation for 2 weeks, the segments were cold-hardened at 4°C for 3 weeks under a 12 h photoperiod at 20 mol $m^{-2}s^{-1}$. Axillary buds of 1 mm in size with 5 leaf primordia were excised from the cold-hardened segments and precultured on basal MS (Murashige and Skoog 1962) medium containing 0.3 M sucrose, 1 mg/l GA_3, 0.01 mg/l BAP and 0.001 mg/l NAA at 23°C for 16 h. Precultured shoot tips were encapsulated in 0.1 M $CaCl_2$ solution containing 0.4 M sucrose and 2 M glycerol, for 30 min at 25 ^{o} C to form beads of about 4 mm in diameter, each bead containing one shoot tip. The beads were osmoprotected with a loading solution composed of MS (Murashige and Skoog 1962) medium supplemented with 2 M glycerol and 0.6 M sucrose, on a rotary shaker (60 rpm) at 25°C for 90 min. Osmoprotected beads were dehydrated with PVS2 on a rotary shaker (45 rpm) at 0°C for 3 h. After dehydration, beads were transferred in 1 ml PVS2 contained in 1.8-ml cryotubes which were then directly plunged into LN. After cryostorage, cryotubes were rapidly warmed in a water bath at 38°C. Frozen shoot tips were washed with 1.2 M sucrose solution for 10 min, followed by post-culture on the basal medium for 1 day and then transferred onto the basal medium containing 0.0005 mg/l GA_3 for recovery. Surviving shoot tips resumed growth within 3 days and developed shoots within 10 days of post-culture without intermediary callus formation. When this encapsulation-vitrification protocol was applied to other 12 cultivars, recovery varied with genotype (**Table 1**).

According to the authors, the encapsulation-vitrification method usually produces much higher shoot regeneration and much faster regrowth than encapsulation-dehydration (Hirai and Sakai 1999, 2000). This method is also easy to handle and can be used to treat a large number of shoot tips at the same time. However, genotype-specific response is still a main problem limiting its wide applications to different genotypes of potato (Hirai and Sakai 1999, 2000).

Table 1 Examples of potato shoot tips cryopreserved by novel cryogenic techniques since 1990.

Cryogenic procedure	Species	Cultivars or accessions	Reference
Encapsulation-vitrification	*S. tuberosum*	14	Hirai and Sakai 1999
	S. tuberosum	13	Hirai and Sakai 2000
	S. tuberosum	1	Wang *et al*. 2006
Encapsulation-dehydration	*S. phureja*	1	Fabre and Dereuddre 1990
	S. phureja	1	Benson *et al*. 1996
	S. tuberosum	1	Benson *et al*. 1996
	S. Brachycarpum	1	Benson *et al*. 1996
	S. acaule	1	Benson *et al*. 1996
	S. guerreroense	1	Benson *et al*. 1996
	S. iopetalum	1	Benson *et al*. 1996
	S. phureja	2	Bouafia *et al*. 1996
	S. phureja × *chacoense*	1	Bouafia *et al*. 1996
	S. tuberosum	2	Bouafia *et al*. 1996
	S. tuberosum	14	Hirai and Sakai 1999
	S. tuberosum	1	Grospietsch *et al*. 1999
	S. tuberosum	12	Hirai and Sakai 2000
	S. tuberosum	1	Wang *et al*. 2006
Droplet freezing	*S. tuberosum*	125	Schäfer-Menuhr *et al*. 1996
	S. tuberosum	219	Schäfer-Menuhr *et al*. 1997
	S. tuberosum	245	Mix-Wagner 1999
	S. tuberosum	10	Barandalla *et al*. 2003
	S. tuberosum	260	Keller and Dreiling 2003
	S. tuberosum	150	Panta *et al*. 2006
	S. tuberosum	4	Kryszczuk *et al*. 2006
	S. tuberosum	5	Keller *et al*. 2006
	S. tuberosum	4	Faltus *et al*. 2006
	S. tuberosum	1	Wang *et al*. 2006
	S. tuberosum	5	Sarkar and Naik 1998
Vitrification	*S. tuberosum*	2	Zhao *et al*. 2005
	S. tuberosum	4	Kryszczuk *et al*. 2006
	S. tuberosum	3	Halmagyi *et al*. 2005
	S. tuberosum	6	Kim *et al*. 2006
Droplet-vitrification	*S. stenotomum*	2	Kim *et al*. 2006
	S. gonicalxy	2	Kim *et al*. 2006
	S. chacoense	2	Kim *et al*. 2006
	S. tuberosum	2	Yoon *et al*. 2006
	S. tuberosum	1	Yoon *et al*. 2007
	S. stenotomum	1	Yoon *et al*. 2007

Droplet-vitrification

The droplet-vitrification method is based on the droplet method described by Schäfer-Menuhr *et al.* (1994, 1996, 1997). *In vitro* stock shoots were maintained on hormone-free MS (Murashige and Skoog 1962) medium at 24°C under a 16 h photoperiod with a light intensity of 39 $\mu Em^{-2}s^{-1}$ (Halmagyi *et al*. 2005). Subculture was done once every 4 weeks. Apical shoot tips of 3-4 mm in length with 2-4 leaf primordia were excised from 1- to 2-month old stock shoots and then placed for 24 h at 24°C on filter papers saturated with P_1 medium made of liquid MS (Murashige and Skoog 1962) medium containing 0.4 mg/l GA_3, 0.5 mg/l zeatin and 0.2 mg/l IAA. Shoot tips were then precultured on MS (Murashige and Skoog 1962) medium supplemented with 0.5 M sucrose. Following preculture for 24 h, each shoot tip was dehydrated in a 4 µl droplet of PVS2 placed on sterilized aluminum foil strips of 0.6 cm × 1.5 cm for 20 min at room temperature. After dehydration, the aluminum foils with shoot tips were transferred into 2 ml pre-cooled cryotubes prior to direct immersion in LN. Thawing was carried out by rapid transfer of the frozen aluminum foils into P_1 medium at room temperature. Thawed shoot tips were post-cultured on P_2 medium, a semi-solid (3.5 g/l agar) P_1 medium, for regrowth. With this protocol, cryopreserved shoot tips resumed growth within 20 days and regenerated shoots within 30 days. The average regrowth of cryopreserved shoot tips of three potato (*S. tuberosum*) cultivars was about 51% (Halmagyi *et al*. 2005).

Kim *et al.* (2006) compared the effects of four cryogenic procedures including droplet-vitrification A (Towill and Bonnart 2003), droplet-vitrification B (Kim *et al*. 2006), droplet (Schäfer-Menuhr *et al*. 1996, 1997) and vitrification (Golmirzaie and Panta 2000) on efficiency of cryopreservation of potato shoot tips. They found that the highest survival was obtained using droplet-vitrification B (Kim *et al*. 2006). The main difference between droplet vitrification A and B lies in warming step. In the former, the cryovials are immersed in a water bath at 40°C for 5 s, and then unloading solution containing 0.8 M sucrose is poured in the cryovial, while in the latter, the frozen foil strips with shoot tips are removed out from the cryovial and immediately plunged in pre-heated unloading solution containing 0.8 M sucrose at 40°C for 30 s, followed by treatment in pre-cooled unloading solution. This optimized protocol was successfully applied to 12 accessions of three potato species (*S. tuberosum*, *S. stenotomum* and *S. chacoense*), with their survivals ranging between 64 to 94%. The vitrification-droplet technique has been routinely employed for long-term preservation of potato genetic resources at the International Potato Center (CIP) in Peru (Panta *et al*. 2006) and at the National Institute of Agricultural Biotechnology in Korea (Kim *et al*. 2006; Yoon *et al*. 2006). Up to 2006, 446 accessions of potato had been cryostored at CIP with their recoveries ranging between 5 and 85% (Gonzalez-Arnao *et al*. 2008), thus indicating that success of droplet-vitrification depends on genotype (Panta *et al*. 2006; Gonzalez-Arnao *et al*. 2008).

ULTRASTRUCTURAL AND HISTOLOGICAL STUDIES

Using the ultra-rapid freezing method described by Grout and Henshaw (1978), Grout and Henshaw (1980) carried

out histological and ultrastructural studies on cryopreserved potato shoot tips. Following cryopreservation, surviving cells were observed scattered throughout the tissues of the apical dome and leaf primordia in the form of single cells or small groups of cells. Massive damage caused by freezing-thaw cycle was associated with rupture of the epidermis of the shoot tips. In the damaged areas, total breakdown of the protoplast and in worst cases, cell wall rupture with loss of the protoplast contents were observed by transmission electron microscopy. Surviving shoot tips showed expansion of the leaf primordia within 7-10 days and formed small plantlets within 6 weeks of post-culture (Grout and Henshaw 1980). Occasionally, plantlets could regenerate following expansion of a single leaf primordium and of a part of the meristem dome. However, no plantlets could regenerate in surviving shoot tips that just developed a portion of a single leaf primordium. These shoot tips only developed large, green, expanded leaflets without further shoot development. Similar results were also observed in shoot tips cryopreserved by encapsulation-dehydration (Benson *et al*. 1996). These data indicate that the leaf primordia, but not the apical meristems, withstood freezing in liquid nitrogen. Using the droplet method developed by Schäfer-Menuhr *et al.* (1994), Kaczmarczyk *et al.* (2006) found that cells in the apical dome of potato shoot tips did not survive following cryopreservation, while a small group of cells in the leaf primordium close to the apical dome did survive and subsequently developed into shoots. The above data obtained with different cryogenic procedures suggest that only small group of cells in potato shoot tips withstand freezing, and these small groups of cells are sufficient for new plant regeneration.

Working with the vitrification method, Golmirzaie *et al*. (2000) carried out ultrastructural studies on cryopreserved shoot tips of four potato genotypes. They found that abnormal changes including abnormal cytoplasm, cell plasmolysis of different degrees and a large number of vesicles were most often observed in cryopreserved shoot tips. Damage related with cytoplasm and cell plasmolysis was negatively correlated with survival and differed largely with genotypes. For example, the most serious damage was observed in cryopreserved shoot tips of the genotype 703838 which gave the lowest survival (6.1%) among the other three genotypes.

FACTORS AFFECTING CRYOPRESERVATION

Treatments involved in the whole cryogenic procedure from preparation of stock cultures to plant regeneration have all been found to significantly influence success of cryopreservation of potato shoot tips. Several key factors are briefly discussed below.

Stock cultures

With the vitrification procedure, Zhao *et al.* (2005) found that cold hardening (10°C) of stock cultures for 3 weeks significantly improved recovery of cryopreserved shoot tips, compared with non-hardened ones. Survival of cryopreserved shoot tips of four potato cultivars by droplet vitrification increased when *in vitro* stock cultures were cold-hardened at 6°C for 3 weeks (Panta *et al*. 2006). However, Hirai and Sakai (1999, 2000) reported that both cold-hardened (4°C for 3 weeks) and non-hardened stock cultures gave similar levels of recovery using encapsulation-vitrification. Cold treatment (21/8°C, day/night) of *in vitro* stock plantlets caused reduction in survival and shoot regeneration of shoot tips cryopreserved by vitrification, while the same treatment improved survival and regeneration using droplet freezing (Kryszczuk *et al*. 2006). With the droplet vitrification procedure, Halmagyi *et al.* (2005) suggested that cold hardening (4°C) was not necessary for successful cryopreservation of shoot tips of three potato cultivars (*S. tuberosum*). Therefore, it is likely that the effect of cold treatment of the stock cultures may depend on the cryogenic method or cultivar used. Subculture duration has been shown to largely influence survival of cryopreserved shoot tips (Bouafia *et al*. 1996; Yoon *et al*. 2006). With *S. tuberosum*, the optimal duration was 5 and 7 weeks for cvs. STN13 and Dejima, respectively (Yoon *et al*. 2006). Culture conditions of the stock shoots including light intensity, aeration and planting density were all shown to significantly influence survival of cryopreserved shoot tips (Yoon *et al*. 2006, 2007). A combination of high light intensity, good aeration of culture vessels and low planting density resulted in high survival. Among these factors, aeration was shown to be the most important one affecting survival (Yoon *et al*. 2006, 2007).

Shoot tips

Halmagyi *et al.* (2005) found that recovery of cryopreserved shoot tips of three potato cultivars (*S. tuberosum*) decreased as bud position increased from the terminal (bud 1) to the basal bud (bud 5). Survival of cryopreserved shoot tips at early stage (3-4 weeks following post-culture) was lower in shoot tips taken from lower part of the stock shoots than from other parts, while the survival at late stage (5-7 weeks following post-culture) was lower in apical shoot tips of the stock shoots than from other parts (Yoon *et al*. 2006). However, Hirai and Sakai (1999) did not observe any additional effects of bud position on survival of cryopreserved shoot tips. Comparing effects of size of shoot tips on survival, Halmagyi *et al.* (2005) demonstrated that size of shoot tips significantly influenced survival with the highest recovery obtained with 3-4 mm shoot tips. Smaller (1-2 mm) or larger (5-6 mm) shoot tips displayed lower survival. Using two cultivars for cryopreservation, Yoon *et al.* (2006) found that optimal size of shoot tips for survival was 1.5-2.0 mm for cv. 'Dejima' and 1.0-1.5 mm for cv. 'STN13'. These data indicate that the optimal size of shoot tips for survival may vary with different cultivars. Reactivation of excised shoot tips, by culturing them on the same medium as used for maintenance of the stock cultures, for 2 weeks before encapsulation, was found to significantly improve survival of cryopreserved shoot tips of *S. phureja* cv. 'Si22' (Bouafia *et al*. 1996).

Preculture

A prerequisite for successful cryopreservation is the induction of a high level of tolerance of shoot tips to dehydration and subsequent freezing in LN. In many cases, preculture is a necessary step to induce such tolerance. Sucrose is the sugar most often used for preculture (Fabre and Dereuddre 1990; Bouafia *et al*. 1996; Grospietsch *et al*. 1999; Hirai and Sakai 1999; Yoon *et al*. 2006), although other sugars such as glucose, mannitol and sorbitol have also been tested (Sarkar and Naik 1998; Halmagyi *et al*. 2005; Criel *et al*. 2006). Two preculture methods, i.e. direct and stepwise preculture, are mainly used. Sucrose concentrations ranging from 0.3 to 0.75 M were usually found suitable for obtaining high survivals of cryopreserved shoot tips (Fabre and Dereuddre 1990; Bouafia *et al*. 1996; Hirai and Sakai 1999; Halmagyi *et al*. 2005). Using direct preculture with various sucrose concentrations ranging from 0.3 M to 1.5 M, Halmagyi *et al.* (2005) found that the optimal concentration for recovery of cryopreserved shoot tips of three potato (*S. tuberosum*) was 0.5 M. Among the four sugars including sucrose, glucose, sorbitol and mannitol tested for preculture, sucrose was found best for shoot regeneration of cryopreserved shoot tips of three cultivars (*S. tuberosum*) (Halmagyi *et al*. 2005). Compared with direct preculture, stepwise preculture with increased sucrose concentration, i.e. 0.3 M sucrose for 24 h followed by 0.7 M sucrose for 7 h, markedly enhanced survival of cryopreserved shoot tips (Yoon *et al*. 2006). Preculture with a mixture of 0.3 M sucrose and 0.2 M mannitol produced much higher survival and direct shoot regeneration of cryopreserved shoot tips than preculture with 0.3 M sucrose alone and higher sucrose concentra-

tions (0.5-0.7 M) (Sarkar and Naik 1998). Preculture with sugar-enriched medium was found to significantly promote accumulation of internal sugars and proline level of precultured shoot tips, which was beneficial to survival of cryopreserved shoot tips of potato (Grospietsch *et al*. 1999). Recently, Criel *et al.* (2006) reported that preculture with sorbitol at 0.055-0.22 M largely improved recovery of cryopreserved shoot tips of potato cv. 'Desiree' (*S. tuberosum*). Proteome analysis revealed different protein patterns between plantlets precultured with sorbitol and without sorbitol (Criel *et al*. 2006). Carbohydrates and polyols were much higher in shoot tips precultured with sorbitol than in the controls. Such high intracellular osmolyte concentrations may contribute to the achievement of high recovery of cryopreserved shoot tips (Criel *et al*. 2006).

Loading

In vitrification-based procedures, exposure of precultured shoot tips to plant vitrification solution without osmoprotection caused in many cases harmful stress to the shoot tips (Sarkar and Naik 1998; Hirai and Sakai 1999, 2000). Thus, an osmoprotection (loading) of the shoot tips was necessary in order to obtain high survival of cryopreserved shoot tips (Sarkar and Naik 1998; Hirai and Sakai 1999, 2000; Zhao *et al*. 2005). Using different loading solutions composed of various sucrose concentrations (0.4-1.6 M) and their combinations with 2 M glycerol for osmoprotection, Hirai and Sakai (1999, 2000) showed that a mixture of 0.4 M sucrose and 2 M glycerol gave the best results of shoot formation of cryopreserved shoot tips. Survival of cryopreserved shoot tips increased markedly when precultured shoot tips were successively loaded with 20% and 60% PVS2 before vitrification with 100% PVS2 (Sarkar and Naik 1998). In contrast to the above results, loading treatment (2 M glycerol and 0.4 M sucrose) did not significantly affect survival of shoot tips cryopreserved by droplet-vitrification (Yoon *et al*. 2006).

Vitrification

Vitrification is the physical transition process of water from a liquid directly into a non-crystalline amorphous phase, a glass, by an extreme elevation in viscosity during cooling (Fahy *et al*. 1984). Although other plant vitrification solutions have been tested, PVS2 has been the most often used one (Sarkar and Naik 1998; Hirai and Sakai 1998; Halmagyi *et al*. 2005; Zhao *et al*. 2005; Kim *et al*. 2006; Yoon *et al*. 2006). Duration of exposure to vitrification solution varied with different species or cultivars (Halmagyi *et al*. 2005) and sizes of shoot tips (Halmagyi *et al*. 2005). Recently, Zhao *et al.* (2005) found that addition of Supercool x1000, a new ice blocking agent, to PVS2 markedly enhanced recovery of cryopreserved shoot tips of two potato cultivars (*S. tuberosum*).

Warming

Both slow warming at room temperature (Schäfer-Menuhr *et al*. 1994, 1996; Bouafia *et al*. 1996; Schäfer-Menuhr *et al*. 1997; Halmagyi *et al*. 2005) and rapid warming at 35-40°C for 1-3 min (Hirai and Sakai 1998; Sarkar and Naik 1998; Zhao *et al*. 2005; Kryszczuk *et al*. 2006) have been employed in cryopreservation of potato shoot tips. Much higher survival of cryopreserved shoot tips was obtained by dipping the frozen foil strips in pre-heated unloading solution (0.8 M sucrose) at 40°C for 30 s than by dipping the frozen foil strips into a water bath at 40°C for 5 s (Kim *et al*. 2006; Yoon *et al*. 2006, 2007).

Unloading

In vitrification-based procedures, unloading is used to dilute the vitrification solution and remove cryoprotectants, which may otherwise cause phytotoxic effects to cells of cryopreserved tissues, thus reducing viability (Steponkus *et al*. 1992). For successful cryopreservation of potato by vitrification-based procedures, unloading is generally performed by incubation of warmed shoot tips with 0.8-1.2 M sucrose solution for 10-30 min (Hirai and Sakai 1998; Sarkar and Naik 1998; Zhao *et al*. 2005; Kim *et al*. 2006; Yoon *et al*. 2006).

Post-culture

As suggested by Bhojwani and Razdan (1996) and Wang *et al.* (2003a), shoot tips following cryopreservation may require a special medium for their survival and plant regeneration in the course of post-culturing. It is common that not all surviving shoot tips are able to regenerate shoots (Grout and Henshaw 1980; Schäfer-Menuhr *et al*. 1996; Benson *et al*. 1996; Schäfer-Menuhr *et al*. 1997; Kaczmarczyk *et al*. 2006). Solid MS medium containing 30 g/l sucrose and supplemented with a combination of plant growth regulators such as those described by Towill (1983), Bouafia *et al* (1996), Sarkar and Naik (1998), and Yoon *et al* (2006) usually are beneficial to survival and plant regeneration. Recently, Zhao *et al*. (2005) reported that addition of low concentration (0.0005%) of Pluronic F-68, a difunctional block copolymer terminating in primary hydroxyl groups, to the post-culture medium significantly improved recovery of cryopreserved shoot tips of two potato cultivars (*S. tuberosum*). A study performed by Kryszczuk *et al.* (2006) showed that shoot tips cryopreserved by droplet freezing regenerated shoots similarly both on solid and in liquid medium. However, regeneration was much lower in surviving shoot tips cryopreserved by vitrification than by droplet freezing, with many of them developing only callus when post-cultured in liquid medium. Post-culture conditions have also been found to affect recovery of cryopreserved shoot tips. A single shoot formed from each cryopreserved shoot tip when post-cultured on solid MS medium supplemented with 0.09 M sucrose and 2.9 μM GA_3 under a 16 h photoperiod of 40 μmol $m^{-2}s^{-1}$ light intensity (Sarkar and Naik 1998). However, multiple shoots developed from each cryopreserved shoot tip when post-cultured on solid MS medium containing 0.2 M sucrose, 5.8 μM GA_3 and 1.0 μM BA under a 16 h diffuse light (6 μmol$m^{-2}s^{-1}$) for the first week, and then transferred to a 16 h photoperiod of 40 μmol $m^{-2}s^{-1}$ light intensity.

GENETIC STABILITY

As stated by Engelmann (1997), cell division and metabolism of plant materials stored in LN cease, and in theory, any genetic variations of materials stored in this way can be avoided. However, since cryopreservation techniques involve not only freezing in liquid nitrogen but also *in vitro* tissue culture and regeneration processes, cryostorage-derived plants may be subject to somaclonal variation induced during these stages, leading subsequently to distinct differences in their genotype/phenotype profiles (Harding 2004). Therefore, it is important to evaluate whether plant materials regenerated from cryostorage are true-to-type to the original stock materials. Until now, genetic stability of potato plants recovered from cryopreserved shoot tips has been evaluated at the morphological (Benson *et al*. 1996; Schäfer-Menuhr *et al*. 1996, 1997; Sarkar and Naik 1998; Hirai and Sakai 1999; Barandalla *et al*. 2003; Halmagyi *et al*. 2005; Keller *et al*. 2006), histological/cytological (Benson *et al*. 1996; Schäfer-Menuhr *et al*. 1996, 1997; Barandalla *et al*. 2003) and molecular levels (Harding 1991; Schäfer-Menuhr *et al*. 1996, 1997; Hirai and Sakai 1999, 2000).

Survival and regeneration remained stable in shoot tips of 51 varieties that had been cryopreserved for several years (Mix-Wagner *et al*. 2003). Keller *et al* (2006) reported that regeneration capability remained unchanged in potato shoot tips that had been cryostored for up to 10 years. Morphologies of plants recovered from cryopreservation of different

potato varieties were identical to those of the control plants (Schäfer-Menuhr *et al*. 1996; Benson *et al*. 1996; Schäfer-Menuhr *et al*. 1997; Mix-Wagner 1999, Hirai and Sakai 1999, 2000; Halmagyi *et al*. 2005; Keller *et al*. 2006). Among the plants regenerated from cryopreserved shoot tips of 98 cultivars, only one plant was abnormal, which was probably due to polyploidy (Schäfer-Menuhr *et al*. 1996, 1997). However, this suspected polyploid regenerant was not verified with flow cytometry (Schäfer-Menuhr *et al*. 1996, 1997). Using flow cytometry and DNA-fingerprinting for determining genetic stability of plants regenerated from cryopreserved shoot tips, Schäfer-Menuhr *et al* (1996, 1997) found that, of the 161 samples of regenerated plants, neither polyploidy nor abnormal banding patterns were found, and no changes in restriction fragment length polymorphisms (RFLPs) were detected. With six potato species including diploid, tetraploid and hexaploid species, Benson *et al.* (1996) found that ploidy status of all plants following cryostorage was stable and no chromosomal abnormalities were observed. Similar results were also obtained in potato plants regenerated from cryopreserved shoot tips (Barandalla *et al*. 2003). No difference was observed in random amplified polymorphic DNA (RAPD) analysis using 17 primers between cryopreserved and the control plantlets (Hirai and Sakai 1999, 2000). Taking all these elements together, the conclusion can be reached that plants regenerated from cryopreserved shoot tips of potato are genetically stable.

VIRUS ELIMINATION

Potato viral diseases constitute a major constraint to sustainable production of potato. *Potato leafroll virus* (PLRV) and *Potato virus Y* (PVY) are among the most popular viral diseases causing serious damages to potato crops (Loebenstein and Manadilova 2003; Valkonen 2007). Recently, cryotherapy of shoot tips has been found to efficiently eliminate plant pathogens such as viruses (Brison *et al*. 1997; Helliot *et al*. 2002; Wang *et al*. 2003b, 2006, 2008; Wang and Valkonen 2008). With potato, Wang *et al.* (2006) attempted to apply three cryotherapy procedures to eliminate PLRV and PVY. Results showed that both PLRV and PVY can be successfully eliminated with high virus-free frequencies of 83-86% and 91-95% obtained for PLRV and PVY, respectively, and no differences were found in efficiency of virus elimination among these three methods. The morphology of the plantlets regenerated from cryo-treated shoot tips was identical to that of non-treated plantlets. In comparison with the traditional methods, frequencies (83-86% for PLRV and 91-95% for PVY) of virus-free plantlets produced by cryotherapy were higher than those obtained by meristem culture (56% for PLRV and 62% for PVY) and thermotherapy (50% for PLRV and 65% for PVY), and similar to those achieved by thermotherapy followed by meristem culture (90% for PLRV and 93% for PVY). Survival (75-85%) and regrowth (83-89%) of cryo-treated shoot tips were higher than those obtained using meristem culture (50-55%) and thermotherapy followed by meristem culture (40-50%), but similar to those achieved using thermotherapy (80-87%). Furthermore, the time period required to implement the whole procedure was shortest for cryotherapy (about 55 days), while about 87 days were needed for thermotherapy and thermotherapy followed by shoot tip culture. Based on the above data, the authors suggested cryotherapy of shoot tips as a simple and efficient method for elimination of potato viruses. For the long-term preservation of potato genetic resources using shoot tips, virus-free stock cultures are required in order to preserve "clean" germplasm (Fabre and Dereuddre 1990; Bouafia *et al*. 1996; Sarkar and Naik 1998; Keller *et al*. 2003, 2006). Thus stock cultures are first subject to treatments of virus elimination and then virus-free cultures are used for cryopreservation. This is troublesome, costly and time-consuming. Because viruses can be efficiently eliminated by cryotherapy of shoot tips, virus elimination from the stock cultures before cryopreservation can be avoided and the materials can be simultaneously prepared for both virus elimination and long-term preservation. After cryostorage, sanitary status of regenerated plants can be tested and virus-free materials be selected. Selection of virus-free materials is expected to be more efficient, since the majority of plants regenerated from cryopreserved shoot tips is virus-free (Wang *et al*. 2006), thus largely reducing the cost and time duration required for virus elimination before cryopreservation.

CONCLUSIONS

Great efforts have been made during the last two decades to establish novel cryogenic procedures suitable for cryopreservation of shoot tips of *Solanum* species. Factors involved in the whole procedures from preparation of *in vitro* stock cultures to plant regeneration following cryopreservation have been extensively studied and found to affect the success of cryopreservation. Through these studies, various cryopreservation techniques have been established including droplet, vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet-vitrification. Genetic stability of plants regenerated from cryopreserved shoot tips was confirmed by studies at the morphological and molecular levels. Routine application of cryopreservation of shoot tips to the long-term conversation of potato genetic resources has already started in Germany, Peru, Czech Republic and Korea. Cryotherapy of shoot tips was found to efficiently eliminate potato viruses, providing an alternative method for virus elimination and giving the additional value that the materials can be simultaneously prepared for long-term preservation. Yet, species- or cultivar-specific is the main difficulty, and has to be taken into consideration when cryo-protocols are to be developed. Fundamental studies on mechanism(s) by which potato shoot tips can withstand freezing in LN, which has received much less attentions in the previous studies, would help develop protocols suitable for cryopreservation of different potato species and cultivars.

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