Plant Cell Tiss Organ Cult (2015) 120:117–125
DOI 10.1007/s11240-014-0585-2

ORIGINAL PAPER

Improved cryopreservation method for the long-term conservation of the world potato germplasm collection

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Received: 20 May 2014 / Accepted: 28 July 2014 / Published online: 13 August 2014

Abstract The effect of cold and sucrose pretreatment for increasing tolerance to cryopreservation was evaluated with eight diverse genotypes, six cultivars belonging to the cultivated species, *Solanum tuberosum* spp., *S. tuberosum* subsp. *andigena*, *S.* x *juzepczukii* and *S.* x *ajanhuiri*, and two genotypes from the wild species, *S. commersonii*. *In vitro* plantlets were cultured at either 6 or 22 °C in media supplemented with either 0.07 or 0.3 M sucrose prior to droplet PVS2 cryopreservation. The sucrose pretreatment appeared to have no positive effect on post-cryo survival. The cold-hardening pretreatment increased significantly post-cryo recovery in drought and frost tolerant cultivars. When 755 accessions, representing 10 taxa, were cryopreserved after cold-hardening, 96 % responded with at least one shoot recovering and 63 % showed a high recovery rate (40–100 %). Therefore this method is recommended for the long term conservation of diverse accessions of potato germplasm.

Keywords Abiotic stress · Andean potatoes · Genebank · Genetic resources collection · PVS2 · *Solanum* spp.

Abbreviations

ARS	Agricultural research service US Department of Agriculture
CIP	International Potato Center
CRI	Crop Research Institute in the Czech Republic
DMSO	Dimethyl sulfoxide
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Statistics report of the FAO
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research, Germany
LN	Liquid nitrogen
LS	Loading solution
MS	Murashige and Skoog (1962) culture medium
NAC	National Agriculture Center in the Republic of Korea
NCGRP	National Center for Genetic Resources Program in the United States
PVS2	Plant vitrification solution 2
RS	Recovery solution

Introduction

Potato has the richest genetic diversity of any staple crop (Messer 2000). In the Andean region, currently more than 4,500 potato landraces belonging to seven *Solanum* species

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exist. Around the world, there are hundreds of improved varieties, derived mainly from *Solanum tuberosum* spp. *tuberosum*. This wide diversity needs to be preserved to secure food security for future generations. These varieties are indeed a rich reservoir of genetic characteristics to improve potato varieties and increase food supply. Cryopreservation is thereby an important tool to ensure the safe preservation of this diversity.

Research on potato cryopreservation was initiated in 1977 (Bajaj 1977) and over time several methods have been developed and assessed demonstrating the advantage of cryopreservation for long-term germplasm conservation. In comparison to in vitro conservation, cryopreservation decreases material handling during storage. Therefore it minimizes labor and risks of losing samples due to human errors. Consequently there is less contamination risk. An efficient cryopreservation procedure allows plant tissues to tolerate the ultralow temperature of −196 °C of liquid nitrogen (LN) and, to withstand this LN storage for an unlimited time period; and it allows the subsequent regrowth of true-to-type plants.

For the safe long-term conservation of important staple clonal crops such as potato, banana, cassava, sweetpotato, yam and other Andean root and tuber crops, international genebanks (Benson et al. 2011) are conducting research on the development of improved cryopreservation methods and the establishment of cryo-banks as part of a global strategy to securely preserve genetic resources into perpetuity. In potato cryopreservation techniques like the PVS2 droplet vitrification and the DMSO droplet method are routinely used. The PVS2 droplet method is used at the International Potato Center (CIP), Lima, Peru, where the largest global in vitro potato collection is maintained, comprising Andean cultivars belonging to ten diverse potato taxa. The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany, is currently cryopreserving its potato collection with the DMSO droplet freezing method (IPK 2013). The IPK collection comprises mainly European commercial improved cultivars belonging to the species *Solanum tuberosum* subsp. *tuberosum*. The National Center for Genetic Resources Program in the United States (NCGRP) (ARS 2013) and the National Agriculture Center (NAC) in the Republic of Korea (Kim et al. 2006) also apply the PVS2 droplet vitrification method to cryopreserve potato. Finally, the Crop Research Institute (CRI) in the Czech Republic is cryo-preserving potato using an alternative ultra-rapid freezing method (Kaczmarczyk et al. 2011).

The aim of the present study was to improve the PVS2 droplet vitrification method in use at CIP (Panta et al. 2006) by testing varying pretreatments that could influence potato tolerance to dehydration and freezing. Literature reviews on potato cryopreservation propose several methods to induce tolerance to dehydration and freezing (Gonzales-Arnao et al. 2008; Wang et al. 2008; Benson et al. 2011; Kaczmarczyk et al. 2011). To enhance plantlet hardiness, cold and sugar treatments have been applied during the culture of mother plantlets from which the shoot tips are excised. In this research, a cold-pretreatment of mother plants is termed "cold-hardening".

Cold-hardening treatments at 4 °C, applied for 3 weeks (Hirai and Sakai 2000) and 1 week (Halmagyi et al. 2005) potato plantlets showed no significant improvement in comparison with the PVS2 vitrification without cold-hardening. However improvement was observed when alternating temperatures [21–22/8 °C (day/night)] were applied for 1 week to improved European potato varieties following the DMSO droplet freezing method (Kryszczuk et al. 2006; Kaczmarczyk et al. 2011).

Sugars (sucrose, glucose, mannitol and sorbitol) have also been applied in the shoot tip culture medium preceding exposure to vitrification or dehydration. Sucrose is the most commonly used to induce cryo-tolerance. Halmagyi et al. (2005) successfully demonstrated increased tolerance to cryopreservation by applying 0.5 M sucrose pre-culture for 24 h at 24 °C. Folgado et al. (2014) demonstrated as well an improved recovery of meristems following an elevated sucrose treatment (0.3 M) during the 2 weeks of cold-hardening of mother plants.

In this study, the culturing of shoot-tip-donor plantlets at a low temperature (6 °C) on a culture medium with or without high sucrose concentration (0.3 M) was evaluated using eight genotypes with a different response to abiotic stresses (frost, drought and salinity) and belonging to different species and ploidy levels (Table 1). Since, tissues are subjected to dehydration, osmotic stress and freezing during cryopreservation, we hypothesize that the cryopreservation response is linked to abiotic stress tolerance and that a better understanding of the basis of such tolerance will allow the development of more efficient cryopreservation procedures applicable to a wide range of potato accessions (Panta et al. 2014).

Recently, the Andean cultivated potatoes and their wild relative *S. commersonii* were biochemically and molecularly studied to understand the physiological mechanisms linked to abiotic stress tolerance (Schafleitner et al. 2007; Mane et al. 2008; Vasquez-Robinet et al. 2008; Pino et al. 2013). Information from these studies is useful to improve potato cryopreservation methods.

Materials and methods

Plant material

The potato clones used in this study were obtained from the in vitro collection maintained under slow growth

Table 1 Potato genotypes used in this study and their abiotic stress tolerance/susceptibility in the field

Genotype[a]	Species (ploidy)	Biological status	Reaction to:[b]		
			Drought	Frost	Salinity
Ury	*S. commersonii* (2*x*)	Wild genotype	N/A	R	N/A
FB 5079.1	*S. commersonii* (2*x*)	Wild genotype	N/A	R	N/A
Ccompis	*S. tuberosum* subsp. *andigena* (4*x*)	Landrace	MT	N/A	N/A
Desiree	*Solanum* spp. (4*x*)	Improved cultivar	MT	S	S
H-1	*Solanum* spp. (4*x*)	Improved cultivar	N/A	R	N/A
Piñaza	*S.* x *juzecpzukii* (3*x*)	Landrace	N/A	T	T
Tacna	*Solanum* spp. (4*x*)	Improved cultivar	MR	N/A	T
Wila Yari	*S.* x *ajanhuiri* (2*x*)	Landrace	N/A	T	N/A

Source: CIP's internal genebank database (Dec, 2012)

[a] Genotype identifiers from CIP's genebank database: Ury = CIP 761092; FB 5079.1 = CIP 721102; Ccompis = CIP 700921; Desiree = CIP 800048; H-1 = CIP 376181.5; Piñaza = CIP 702445; Tacna = CIP 390478.9; Wila Yari = CIP 706210

[b] Reaction to drought, frost or salinity: *N/A* no data available, *MR* moderate resistant, *MT* moderate tolerant, *R* resistant, *S* susceptible, *T* tolerant

conditions at CIP. The medium contained 4 % sorbitol, MS salts (Murashige and Skoog 1962), 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.4 mg/l thiamine, 2 % sucrose and 0.8 % agar (SIGMA A-7002). Storage of the cultures took place at 6–8 °C under 22 µmol m^2/s illumination with a 16 h photoperiod. Eight genotypes with differential responses to abiotic stress (drought, frost and salinity), were selected (Table 1). Plantlets were multiplied using single-node cuttings cultured in Magenta® GA-7 vessels containing MS propagation medium (MPM) [MS salts supplemented with 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.1 mg/l thiamine, 25 g/l sucrose and 2.8 g/l Phytagel (SIGMA P-8196)] and held at 22 °C with 45 µmol m^2/s illumination and a 16 h photoperiod. For experimental treatments, shoots were multiplied by culturing 20 apical cuttings (~0.5–0.7 cm length) per GA-7 vessel.

Excision of meristems and sample preparation

Apical cuttings (~0.5 cm) were excised from 3 week old in vitro plantlets. Leaves were removed and the stem was trimmed until an apical shoot tip (1.8–2.5 mm long) comprising the meristematic dome plus 4–5 primordial leaves was dissected. When the shoot tip was larger than 2.5 mm, the tip of the largest primordial leaf was cut reducing the shoot size to the desired length. Sixty to one-hundred-twenty shoot tips, depending on the number of samples required for the experiment, were excised and placed on pieces of sterile filter paper (1.5 cm^2) on potato meristem medium (MMP: MS salts supplemented with 0.04 mg/l kinetin, 0.1 mg/l gibberellic acid, 0.07 M sucrose and 2.8 g/l Phytagel). Ten shoot tips were placed per filter paper in a randomized way when excised to avoid any bias from the length of time post excision (all shoot tips were excised within 1 to 2 h). Samples were incubated at room temperature for about 1 h before the application of the osmoprotective treatment.

Vitrification using the droplet PVS2 method

The shoot tips were rinsed off the filter paper in 5 ml loading solution (LS) (2 M glycerol and 0.4 M sucrose), and incubated in the LS for 15 min at room temperature. The LS was then removed with a Pasteur pipette and replaced with 2 ml ice-cooled filter-sterilized PVS2 (30 % glycerol, 15 % ethylene glycol and 15 % DMSO, dissolved in MS salts with 0.4 M sucrose, pH adjusted to 5.8) for 50 min on ice. Two minutes before the end of the PVS2 treatment period, 10 shoots were transferred to a PVS2 drop (10–15 µl) on an aluminum foil strip (0.5 × 2 cm). All manipulations of the strips were done in a Petri dish placed on ice to obtain a temperature of around 0 °C following the method developed for banana (Panis et al. 2005) and adapted to potato (Panta et al. 2006); the strip holding the shoots was then rapidly plunged into a LN filled cryotube.

Warming and unloading

After one hour storage in LN, aluminum foil strips holding the shoot tips were removed from the LN one by one and rapidly dropped into 4 ml of the Sakai's unloading solution (RS) [MS salts enriched with 1.2 M sucrose (pH 5.8), sterilized by filtration] (Sakai et al. 1990) in a glass vial. Shoots were rinsed and the RS was replaced with fresh RS (~4–5 ml) and incubated 15–20 min at room temperature in the dark.

Post-cryo culture and regeneration

Shoot tips were transferred to filter paper placed on meristem medium (MMP) (MS salts, 0.04 mg/l kinetin 0.1 mg/l gibberellic acid, 2.8 g/l phytagel) with 0.3 M sucrose. Shoot tips were maintained in the dark on a same medium (MMP) but with daily culturing onto fresh medium and decreasing sucrose levels (daily transfers from 0.3, to 0.2, to 0.1 M and finally maintained on 0.07 M). One week after warming, shoot tips were transferred from the filter paper directly onto fresh MMP (0.07 M sucrose) and incubated at 22 °C, with 45 μmol m^2/s illumination for 16 h per day.

Effect of cold and sucrose pretreatment

Plantlets of eight genotypes, 6 cultivars belonging to cultivated species (*Solanum tuberosum* spp., *S. tuberosum* subsp. *andigena, S.* x *juzecpzukii* and *S.* x *ajanhuiri*, and two genotypes from a wild frost tolerant species *S. commersonii*, were cultured as follows: (1) MS medium, at 22 °C; (2) MS medium + 0.3 M sucrose, at 22 °C; (3) MS medium, at 6 °C; and (4) MS medium + 0.3 M sucrose, at 6 °C. After 3 weeks, shoot tips were dissected and processed following the cryo-protocol outlined above. LN-exposed and non-LN exposed controls of each accession were processed. Two replicates of 10 shoot tips per treatment were evaluated in three independent experiments. Survival and recovery rates were recorded 60 days after warming.

Application of cryopreservation to a wide variety of germplasm

Between January 2007 and March 2012, 755 potato accessions were cryo-processed in CIP's genebank applying the PVS2 droplet method using a hardening treatment at 6 °C and a 0.07 M sucrose medium. Twenty out of the frozen samples (70–100) were thawed and their viability evaluated. These together with 20 non-frozen samples were cultured following the protocol described previously but using a modified MMP [MS medium (Murashige and Skoog 1962) with 10 ml/l coconut milk, 20 g/l sucrose, and 2.8 g/l phytagel]. Survival and recovery were recorded 60 days after thawing.

Survival and recovery evaluation

Viability rates, explant survival (survival) and plants recovered (recovery), were evaluated by counting the number of explants showing a green color, and explants growing into green shoots, respectively. The recovered shoots size was about 0.5 cm or longer. Results were recorded 60 days after thawing.

Statistical analysis

Results are presented as mean percentage. The experiment for measuring the cold and sugar effect after cryopreservation was analyzed following a completely randomized design (CRD). Using R and Excel programs, statistical differences between mean values were assessed by ANOVA, LSD or Kruskal–Wallis tests, according to the data normality and variance homogeneity. Survival and recovery were proportional values expressed as percentages. Since proportional data are binomially distributed, they were transformed with arcsin to reach a normal distribution. Prior to transforming the proportional data, values of 0 % were substituted by 1/4n and 100 % by (100-1/4n), where "n" is the number of units on which the percentage data was based. Arcsin formula utilized was y' = arcsin (y/100)1/2 multiplied by 180/π, to convert data to angular grades.

Results

Effect of cold and sucrose hardening on cryo-response

Under cold conditions rooting and shoot growth were reduced. With the 0.3 M sucrose treatment, plantlets showed a reddish color and were elongated; leaves did not fully expand. The cv. Wila Yari was the most affected, as the rooting and growth was the lowest (data not shown). The pretreatment at 6 °C in combination with 0.3 M sucrose medium produced almost no roots. Again plantlets were reddish and leaves not fully expanded. Shoots survived after cryopreservation with significant effects of cultivar ($P < 0.001$) and temperature ($P < 0.05$) (Table 2); however the response to the two sucrose concentrations was similar, overall mean was 69 and 66 % for 0.07 and 0.3 M sucrose, respectively (data not shown). In contrast, for shoot recovery, cultivar, temperature, and also sucrose were highly significant ($P < 0.001$). Moreover a significant interaction of cultivar with temperature was observed ($P < 0.01$). Analysis of this interaction showed that hardening at the low temperature had a positive or non-significant effect on recovery; overall recovery was 48 % with 6 °C and 36 % with 22 °C pretreatment (data not shown).

Results from the individual treatments (Fig. 1) showed that both *S. commersonii* genotypes were highly tolerant to cryopreservation with no significant effect of either cold or sucrose hardening. The cultivars Ccompis, H-1, and Piñaza showed more than 50 % recovery with cold-hardening. The cv. Piñaza was significantly affected by the sucrose (0.3 M)

Table 2 ANOVA on response (shoot survival and recovery after 60 days post-cryo culture) of 8 potato genotypes following cryopreservation after 4 hardening pretreatments

	Df	Sum sq	Mean sq	F value	Pr (>F)
Shoot survival					
Cultivar[a]	7	52,061	7,437.2	18.2504	<2.00E−16***[b]
Temp	1	1,751	1,751	4.2969	0.03978*
Sucrose	1	335	335.5	0.8232	0.3656
Cultivar:temp	7	1,523	217.6	0.5339	0.80791
Cultivar:sucrose	7	4,409	629.8	1.5456	0.15555
Cultivar:temp:sucrose	8	2,027	253.4	0.6217	0.75873
Residuals	160	65,202	407.5		
CV = 29.86					
Shoot recovery (arcsin transformed)					
Cultivar	7	18,964.6	2,709.23	30.1119	<2.20E−16***
Temp	1	2,891.2	2,891.25	32.1349	6.55E−08***
Sucrose	1	1,245.5	1,245.46	13.8427	0.0002747***
Cultivar:temp	7	2,136.9	305.27	3.393	0.0020941**
Cultivar:sucrose	7	694.9	99.27	1.1033	0.3635833
Cultivar:temp:sucrose	8	1,039.7	129.96	1.4445	0.1818899
Residuals	160	14,395.5	89.97		
CV = 34.78					
CV = 23.97	(with arsin transformation)				

Data are from 60 shoot replicates per treatment, i.e. 20 samples in each of the 3 independent experiments. Data were normal (Shapiro test) and variance homogenous (Bartlett test)

[a] 8 potato genotypes: see Table 1

[b] * $P > 0.05$; ** $P > 0.01$; **** $P > 0.001$

treatment. Without cold treatment, Desiree and Wila Yari were more sensitive to cryopreservation irrespective of the sucrose treatment. Tacna showed the lowest recovery rate and none of the treatments increased its recovery, indicating this cultivar was very sensitive to the cryo-procedures applied or not responsive to cold-hardening. These results suggest that a pretreatment with a high sucrose (0.3 M) concentration had no or a negative effect on recovery. However, with cold treatment and 0.07 M sucrose, overall recovery was 52 %, significantly higher than the 40 % obtained with the control treatment (22 °C and 0.07 M sucrose) (data not shown). Moreover, we observed that on average 26 % of the samples (data not shown) survived cryopreservation but failed to re-grow into whole plants.

Based on the results of these experiments a cold-hardening (6 °C) culture phase was included as part of the standard PVS2 droplet vitrification protocol that will be used at CIP. This method that was successful for the most genotypes tested is schematically presented in the Fig. 2.

Application of cryopreservation to a wide range of germplasm

From the beginning of 2007 up to the first quarter of 2012, 755 potato accessions representing the diversity of Andean potato accessions were processed for cryopreservation. For this, the PVS2 droplet method developed previously (Panta et al. 2006) was applied with a cold-hardening treatment at 6 °C in combination with 0.07 M sucrose medium. Results (Table 3) show that 96.4 % of accessions, belonging to 10 potato taxa, responded with at least one shoot recovering after cryopreservation; 50–80 samples of surviving accessions were cryo-stored for the long term. Shoots re-growth was always direct without intermediate callus formation. The overall survival and recovery rates were 75 and 51 %, respectively. Hence, the percentage of surviving shoots that were not able to re-grow into plantlets was 24 %; a rate that is similar to our previous experiments (see above). When the frequency of accessions showing a recovery of 0, 5–39 and 40–100 % was calculated, results showed that 62.9 % of the accessions responded with a shoot recovery higher than 40 %. Accessions belonging to *S. tuberosum* subsp. *tuberosum* and two species of known frost tolerance, *S. chaucha* and *S.* x*juzecpzukii,* showed the highest recovery rates of 76, 65 and 64 %, respectively. However the number of accessions processed for these groups is low due to the low number of accessions in the CIP collection. The most represented species in CIP's collection, S. *tuberosum* subsp. *andigena* showed 51 % recovery and the lowest rate (41 %) was observed for the improved varieties (*Solanum* spp.). In all species, excepting *Solanum* spp., more than 50 % of accessions showed recovery of 40–100 %; and again to *S. tuberosum* subsp. *tuberosum* and the frost tolerant species, *S. chaucha* and *S.* x*juzecpzukii,*comprised the highest percentage (81-86) of accessions with high recovery.

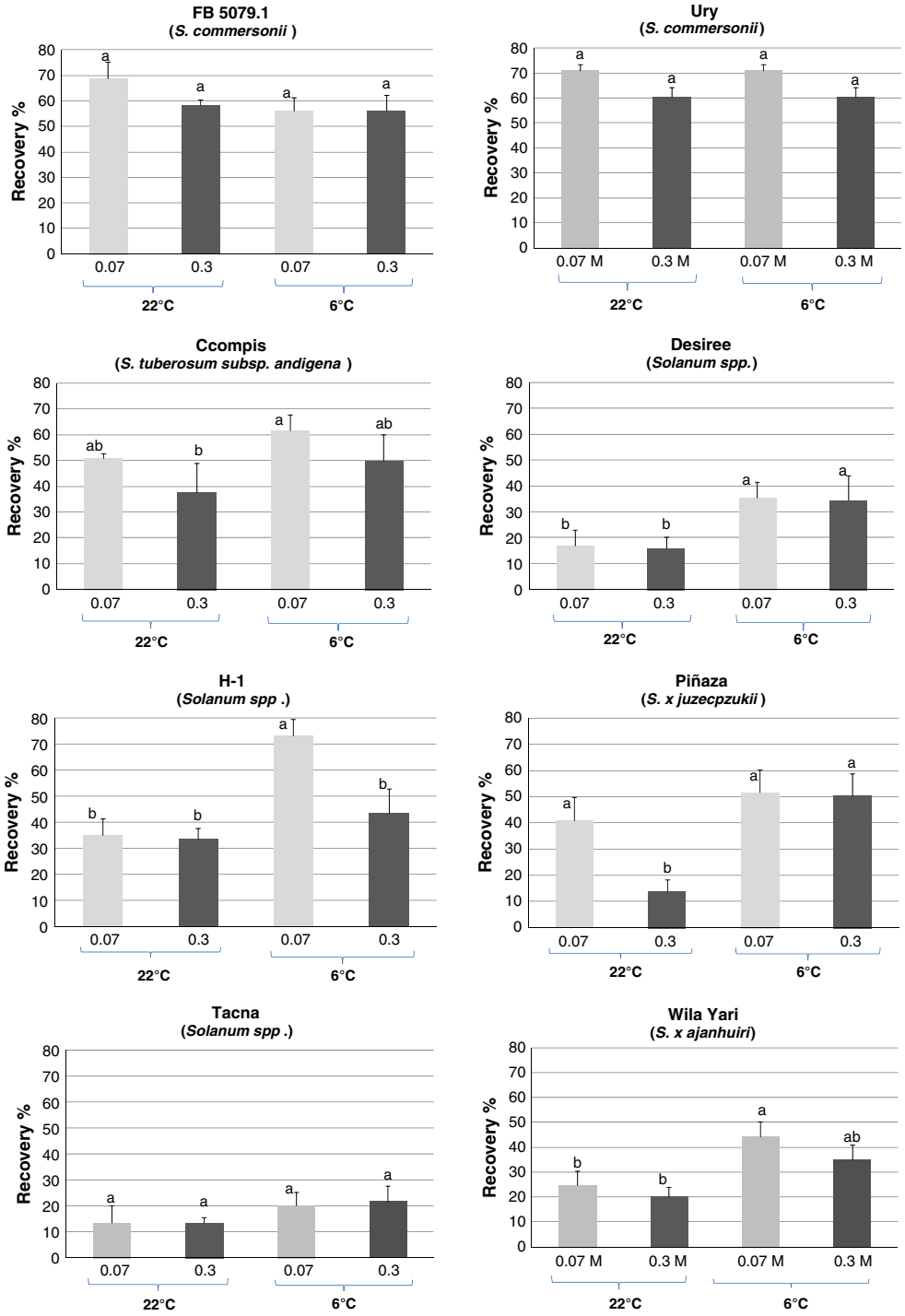

Fig. 1 Effect of temperature (6 and 22 °C) and sucrose (0.07 and 0.3 M) hardening treatments on post-cryo shoot recovery of 8 potato genotypes following a PVS2 droplet vitrification treatment (15 min LS, 50 min PVS2)

Discussion

Research has demonstrated that the addition of cryoprotectants alone often do not provide enough protection against lethal cryo-damage. Both cold and sucrose hardening treatments, either alone or in combination, have been shown to have positive effects on cryopreservation tolerance in grass, hops, yams and potato (Chang et al. 2000; Reed et al. 2003; Leunufna and Keller 2005; Folgado et al. 2014).

This study has shown that a pretreatment of potato shoot-tip-donor-plantlets at 6 °C, followed by droplet PVS2 vitrification is efficient for increasing recovery after cryopreservation in most cultivars tested. The effect of cold pre-culture in PVS2 droplet vitrification has previously been studied in potato (Halmagyi et al. 2005; Hirai and

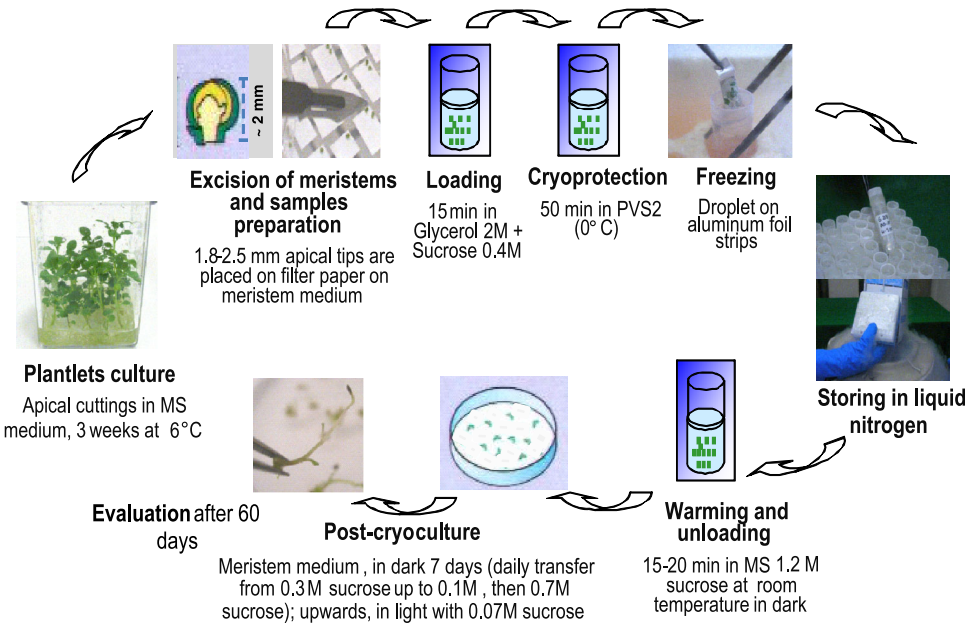

Fig. 2 Schematic representation of an improved vitrification protocol for potato cryopreservation using cold-hardening pretreatment and the droplet PVS2 method. For details see "Materials and methods"

Table 3 Cryopreservation of 755 potato accessions at the International Potato Center (Jan 2007–March 2012)

Species	Number of accessions	Overall survival (S) and recovery (R) (mean %)		Grouping of accessions based on shoot recovery (%)		
		S	R	0	5–39	40–100
Solanum tuberosum subsp. *andigena*	558	73.9	51.1	3.2	20.3	63.4
S. phureja	69	83.4	55.4	1.4	24.2	63.8
S. stenotomum subsp. *stenotomum*	49	71.4	42.5	14.3	17.5	53.1
Solanum spp.	25	69.5	40.7	0	21.3	44.0
S. stenotomum subsp. *goniocalyx*	19	73.6	52.5	0	23.1	63.2
S. chaucha	17	80.7	64.6	6.3	23.8	81.3
S. tuberosum subsp. *tuberosum*	7	86.2	75.5	0	35.0	85.7
S. x*juzecpzukii*	7	94.3	64.3	0	35.0	85.7
S. ajanhuiri	2	92.5	55.0	0	30.0	50.0
S. curtilobum	2	82.5	57.5	0	–	100
Total	755	75.0	51.3	3.6	33.5	62.9

Cryopreservation was done by droplet PVS vitrification (15–20 min LS, 50 min PVS2) with a cold-hardening pretreatment at 6 °C

Sakai 1999). These authors used a pre-culture temperature of 4 °C and the results did not show a significant improvement in comparison with control treatments. The cultivars tested in these studies were improved varieties with no or low acclimation ability. Other studies, using pre-culture at alternating temperatures have demonstrated that cold pre-culture of 7 days at 21 or 22/8 °C (day/night temperature), resulted in an improved recovery rate (Kryszczuk et al. 2006; Kaczmarczyk et al. 2008) when a DMSO droplet method is applied.

We tested cold and sucrose hardening treatments either alone or in combination on accessions of known drought, frost and salinity tolerance such as the frost tolerant cultivars H-1, Piñaza, Wila Yari and two genotypes belonging to *S. commersonii*, the most frost hardy wild potato species known (Bamberg et al. 2005). Also Ccompis, a cultivar that is highly utilized in the Peruvian and Bolivian Andes and of known drought tolerance but from which the reaction towards frost is not clear and the two improved drought tolerant cultivars, Desiree and Tacna, that are unable to cold acclimate, were tested.

It was expected that the frost tolerant genotypes would respond positively to cryopreservation and that the sucrose treatment would enhance this response regardless their cold-acclimation ability. Both the *S. commersonii* frost tolerant genotypes showed a high capacity to recover from cryopreservation regardless the pre-culture temperature or sugar doses applied, suggesting that the ability for tolerating cryopreservation is due to their genetic characteristics instead of the hardening treatments. Studies have demonstrated that fully acclimated *S. commersonii* genotypes can withstand −10 °C (Bamberg et al. 2005) and hence it is not surprising to see such a high cryo-tolerance of this species.

The frost tolerant cultivars, H-1, Piñaza, and Wila Yari always responded better following the cold treatment

(6 °C), but the high sucrose dose (0.3 M) resulted in lower recovery rates. This pattern was similar in Ccompis suggesting that this cultivar might also have a capacity for cold-acclimation. The other improved cultivars, Desiree and Tacna, both having no cold acclimation abilities responded with lower recovery rates than the landraces. Regarding the sucrose effect, our results were different to the positive effect recently reported by Folgado et al. (2014). Authors attribute these differences to the different culture techniques utilized for applying the sucrose treatment. In this study cultures were subjected to the cold and sugar stress immediately after stem cuttings are excised while Folgado's method applied the sucrose treatment in plantlets on 1 week cultures without an excision phase thus preventing an additional cutting stress that can result in the production of stress proteins such as ASRs (Henry et al. 2011).

Cold-acclimation is used in frost tolerance studies and sugar treatments have been applied in drought tolerance research. Plant response to frost and drought implies biochemical and molecular changes. Changes in the amount and composition of membrane lipids may protect against freezing-induced injury. A drought response is mainly related to the synthesis of solutes needed for osmotic adjustment such as soluble sugars, proline, and glycine betaine. In potato, several studies have revealed the stress protecting role of the lipids in cell membranes (Palta 1994), the accumulation of several sugars and osmoprotectant substances (Mane et al. 2008; Evers et al. 2010), as well as genes and proteins for osmotic adjustment (Schafleitner et al. 2007; Carvallo et al. 2011; Pino et al. 2013), and membrane stability and oxidative homeostasis (Vasquez-Robinet et al. 2008; Folgado et al. 2014).

Research on *S. commersonii* and an improved cultivar "Red Pontiac" of *S. tuberosum*, demonstrated that freezing tolerance in the non-acclimated stage and capacity to cold acclimate are following different physiological mechanisms (Palta et al. 1993; Palta 1994). Lipids associated with freezing tolerance in the non-acclimated state are different than those associated with increases in freezing tolerance during cold acclimation. Recently, we have shown that linoleic acid content is positively correlated to cryotolerance in freeze tolerant genotypes (Panta et al. 2009).

The present study clearly highlights the large difference between survival and recovery rates. In all cases the recovery rate was much lower compared to survival rate with an overall decrease from survival to recovery of about 25 %. This emphasises the need for the development of cryopreservation protocols based on shoot recovery rates and not solely on tissue survival.

Cryopreservation recovery in 80 % of the cultivated potatoes by using a cold pretreatment, paves the way for testing and applying cold acclimation on a larger scale (Panta et al. 2011). We tested this optimized method on 755 accessions and observed that the overall recovery rate was 51 %. Only four cultivars did not respond to the cryopreservation procedure, and the best response came from genotypes belonging to frost tolerant species.

We demonstrated that the droplet PVS2 vitrification method preceded by a pre-culture treatment at 6 °C is suitable for application of cryopreservation for the long-term conservation of a wide diversity of potato genotypes. We also concluded that the potatoes with cold-acclimation capacity or frost tolerance are more able to respond positively to cryopreservation.

Acknowledgments The authors want to gratefully acknowledge the Belgian Technical Cooperation (BTC) support for the collaborative work between CIP and the Catholic University of Leuven (KU Leuven); the World Bank for funding part of the research through the Global Public Good project for upgrading the CIP Genebank; and the support from the CGIAR Research Programmes (CRP) Roots, Tubers and Bananas CRP and The Genebank CRP. The authors thank the technical assistance from Edwige André, Karen Reyniers (KU Leuven) and Rosalva Villagaray, Wendy García and Dino Sanchez (CIP).

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