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Unravelling the effect of sucrose and cold pretreatment on cryopreservation of potato through sugar analysis and proteomics

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ABSTRACT

Apical shoot tips were dissected from donor plants (cultured in several conditions) and cryopreserved using the droplet-vitrification technique. The effect of two preculture treatments (sucrose pretreatment medium or cold-culturing during two weeks) on donor plants of four potato species (*Solanum commersonii, S. juzepcukii, S. ajanhuiri,* and *Solanum tuberosum*) was studied. Post-cryopreservation meristem growth and plant recovery were influenced by the treatments, but the effect on the regeneration was strongly genotype-dependent. The highest post-rewarming plant recovery percentage was obtained using meristems dissected from donor plants of *S. commersonii* cultured on sucrose pretreatment medium or cold-cultured. Both preculture conditions also enhanced plant recovery in *S. juzepcukii* compared to control cultures. Cold preculture, however, proved to be undesirable for *S. tuberosum* whereas sucrose pretreatment had a positive impact on the plant regeneration of this species. The determination of changes in the concentration of soluble sugars revealed sugar accumulation, especially of sucrose and the raffinose family of oligosaccharides (RFOs), which can be linked to tolerance towards the cryopreservation. Additionally, a study of the proteome of the donor plantlets after the pretreatments by 2D-fluorescence difference gel electrophoresis (DIGE) was carried out to identify differentially abundant proteins. Carbon metabolism-related proteins, together with stress-response and oxidative-homeostasis related proteins were the main class of proteins that changed in abundance after the pretreatments. Our results suggest that oxidative homeostasis-related proteins and sugars may be associated with the improved tolerance to cryopreservation and the ability to cold acclimate by *S. commersonii* in contrast to the other genotypes. The increased accumulation of sucrose and RFOs play a fundamental role in the response to stress in potato and may help to acquire tolerance to cryopreservation.

1. Introduction

Cryopreservation of plant cells, tissues and organs represents an ideal alternative for the safe, long-term and cost-effective conservation of plant genetic resources [18,19,38]. The plant material can be stored for long periods while the stability of the genetic material is preserved, and phytopathological and physiological risks associated with "classical" methods for the maintenance of plant gene banks are reduced or eliminated [52]. Numerous cryopreservation studies were carried out on potato since Bajaj developed an (ultra)-rapid freezing method [3,35]. Protocols such as vitrification, droplet freezing, encapsulation-dehydration and encapsulation-vitrification have all been applied to potato germplasm (for review see Kaczmarczyk et al. [35]). Though some of these protocols have had a significant success and are now used on a large scale, further studies are still needed to achieve improvement of the plant regeneration.

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The droplet vitrification technique is a widely used method for the cryopreservation of shoot tips in many species including potato [53,59]. Its application on potato germplasm improved the recovery rates of apices in some potato varieties [28,41,54]. Many factors affect the success of cryopreservation of potato shoot tips, such as the physiological state of donor cultures, the type of shoot tips used (apical, axillary), preculture, and cooling and rewarming rates.

Exposure of shoot tip donor plants to sucrose pretreatment medium and cold prior to cryopreservation are often used to improve the recovery results [13,37,43,56]. These abiotic stresses trigger a series of plant responses, thereby preparing the shoot tips for subsequent cryopreservation. Stress perception leads to activation of signaling pathways and alteration of gene expression levels, consequently altering plant physiology, growth and development [23,75]. One of the mechanisms to minimize the deleterious effects caused by abiotic stresses is the synthesis of osmolytes, some of them known to be cryoprotectants. The determination of biochemical changes associated with the cryopreservation ability of plants is, therefore, a useful approach to optimize cryopreservation protocols [36]. In several studies, the biochemical characterization of potato tissues submitted to abiotic stress has already been performed [21,24,57]. Other reports analyzed the changes in protein abundances associated with preculture conditions prior to cryopreservation using potato as a model [16,34]. In our previous work [25] we correlated cryopreservation efficiency and abiotic stress response of two potato species, *Solanum commersonii* and *Solanum tuberosum* 'Désirée'. However, a study involving more genotypes is necessary to develop an improved cryopreservation technology of potential use for the conservation of the entire potato germplasm.

In the current work, we studied the effect of two precultures (sucrose pretreatment medium and cold hardening) on the soluble sugars content and the proteome and their possible implications for the tolerance towards cryopreservation in four potatoes (the commercial cultivar *S. tuberosum* 'Désirée', the wild potato *S. commersonii*, and the two landraces *S. juzepcukii* 'Piñaza' and *S. ajanhuiri* 'Wila Yari').

2. Material and methods

2.1. Plant material and pretreatments

In vitro plantlets of four potatoes accessions [the wild potato *S. commersonii* (CGN18024; provided by the CGR, The Netherlands), the two landraces *S. juzepcukii* 'Piñaza' (CIP702445) and *S. ajanhuiri* 'Wila Yari' (CIP702650) and the commercial cultivar *S. tuberosum* 'Désirée' (CIP800048) (provided by CIP, Peru)] were cultured onto Murashige and Skoog (MS) basal medium [49] (MS salts and vitamins; Duchefa, Haarlem, the Netherlands) with 0.09 M sucrose at 22 °C, 16/8 h day/night and a light intensity of 50 μmol m^{-2} s^{-1}. Ten mm-sized apical shoots were excised from vegetatively-propagated three-week-old *in vitro* plantlets and placed under control conditions (same conditions as above) for one week, before the exposure to the treatments. Thereafter the plantlets were transferred onto a fresh medium and cultured for two weeks under control conditions (same light, temperature and culture conditions as above), or one of the two following pretreatment conditions; (i) the sucrose pretreatment was provided by MS basal medium with 0.3 M sucrose under the same light and temperature conditions, while (ii) plants exposed to the cold were cultivated on MS basal medium with 0.09 M sucrose under the same light conditions but at a constant temperature of 6 °C. All media were solidified with 0.8% agar (Duchefa), and pH was adjusted to 5.8 before autoclaving.

After 14 days of exposure, the growth parameters, length of shoot and number of leaves, were recorded. Additionally, fresh and dry weight (FW and DW) were measured to calculate the water content (WC) of the shoots (calculated as % of water that is present in the shoot, WC = (FW−DW/FW) × 100). The data presented (Table 1) are the mean values of at least three independent experiments with 12 explants per treatment. Data were analyzed using one-way ANOVA, and comparisons between the mean values were evaluated by the least significant different test at $p < 0.05$. The Kolmogorov–Smirnov test was used to verify the normality of the samples, using the SigmaStat software (Systat Software, San Jose, CA).

Shoots from the precultured *in vitro* plantlets were collected and stored at −80 °C. Five biological replicates per treatment, each composed of shoots grown in the same culture container, were sampled for both protein (eight shoots per sample) and sugar (four shoots per sample) analyses. Additionally, shoot tips were excised from plantlets exposed to the three treatments (control and two pretreatments) and subjected to the droplet-vitrification procedure.

2.2. Cryopreservation by droplet-vitrification method

After fourteen days of exposure to the three treatments (control, sucrose pretreatment and cold treatment), apical shoot tips of 1 × 0.5 mm were excised under a binocular microscope. The dissected shoot tip contained the apical dome covered by two leaf primordia and protected at the base by stem tissue. The excised shoot tips were placed in Petri dishes, containing the same preculture medium until all of them were dissected, to prevent tissue dehydration.

The shoot tips were subjected to the droplet-vitrification procedure based on a previously described protocol [51]. Shoot tips were placed into 25 ml plastic vessels, containing a loading solution (LS; filter sterilised solution containing MS basal liquid medium with 2 M glycerol and 0.4 M sucrose). The loading lasted for 20 min in the dark, at room temperature. After loading, the LS was replaced by plant vitrification solution 2 cooled at 0 °C (PVS2; filter sterilized solution consisting of MS basal liquid medium with 3.26 M glycerol, 2.42 M ethylene glycol (EG), 1.9 M dimethyl sulfoxide (Me_2SO) and 0.4 M sucrose) [58]. This dehydration step lasted for 50 min and was done on ice. Five minutes before the end of the treatment, shoot tips were transferred onto an aluminum strip (0.5 × 2 cm) with a plastic Pasteur pipette. The aluminum strip was placed in a Petri dish in contact with a frozen cooling element (a standard PVP pack of 20 × 10 × 1.5 cm, to keep the temperature of meristems and

Table 1
Morphological parameters and water content of three-week-old *in vitro* shoots of four potato genotypes submitted to different stress treatments for 2 weeks (control; sucrose; cold).

Genotype	Treatment	Increase in shoot length (mm)		Number of leaves		Water content (%)	
		Average	StDev	Average	StDev	Average	StDev
Com	Control	72.21[a]	±20.34	6.67[a]	±1.31	92.85[a]	±0.72
	Sucrose	16.91[b]	±6.86	4.92[b]	±1.27	81.84[b]	±2.49
	Cold	11.19[b]	±3.71	3.75[c]	±0.60	77.74[b]	±5.13
Pin	Control	62.03[a]	±13.52	6.64[a]	±0.96	91.24[a]	±2.46
	Sucrose	24.90[b]	±11.70	5.81[b]	±1.58	83.36[b]	±4.74
	Cold	14.40[c]	±4.92	3.97[c]	±0.61	86.90[b]	±3.66
Wya	Control	31.74[a]	±10.01	5.39[a]	±0.99	94.91[a]	±0.52
	Sucrose	11.43[b]	±5.94	3.86[b]	±0.83	84.28[b]	±4.13
	Cold	13.05[b]	±7.89	4.09[b]	±1.12	88.74[ab]	±1.04
Des	Control	43.26[a]	±12.75	6.28[a]	±1.14	92.90[a]	±2.67
	Sucrose	20.63[b]	±5.49	4.51[b]	±0.85	86.36[b]	±3.31
	Cold	15.52[b]	±6.00	4.03[b]	±0.81	85.45[b]	±1.50

Four potato genotypes are: *S. commersonii* (Com), *S. juzepcukii* 'Piñaza' (Pin), *S. ajanhuiri* 'Wila Yari' (Wya) and *S. tuberosum* Désirée (Des). Average and standard deviation (StDev) are indicated.
*Different letters per parameter and genotype indicate values significantly different at $p \leq 0.05$ (ANOVA test).

PVS2 solution around 0 °C during manipulations). After the PVS2 treatment, aluminum strips were plunged into liquid nitrogen and then transferred into 2 ml cryotubes that had previously been filled with liquid nitrogen. For each cryopreservation experiment, some shoot tips were not exposed to liquid nitrogen (non-frozen shoot tips), and they were directly submitted to recovery procedure after the PVS2 treatment.

The cryovials were kept in liquid nitrogen for at least 30 min before rewarming. For rewarming, aluminum strips were removed from the tubes and immediately rinsed in 15 ml unloading solution (US; filter sterilised solution consisting of MS basal liquid medium with 1.2 M sucrose) in a small Petri dish at room temperature. After 5 min, the US was renewed, and meristems were kept for another 15 min in the US. After rewarming, the meristems were picked up from the US and placed on sterile filter paper in Petri dishes with MS basal medium with 0.3 M sucrose solidified with 0.25% gelrite (Duchefa). After one day, the meristems were transferred to Petri dishes with MS medium with 0.09 M sucrose and solidified with 0.25% gelrite, without filter paper.

For the first seven days, the meristem culture was kept in the dark. The shoot tips were observed on a weekly basis under a binocular microscope for at least four weeks. Several types of reactions were distinguished: 1) death of shoot tips; 2) survival: meristems were alive but sometimes the apical dome did not grow out, and only growth of primordial tissues was observed; 3) meristem growth: the growth of a part or the whole meristem; 4) plant recovery: regeneration into a complete *in vitro* plant, with shoot and root regeneration.

The results are presented as mean percentages with their standard deviation. Data were arcsine transformed and analyzed using the two-way ANOVA with the least significant difference and pairwise multiple comparison procedures using Student-Newman-Keuls (SNK) method at $p > 0.001$, using the XLStat software [http://www.xlstat.com].

2.3. Analysis of carbohydrates: extraction and quantification

Carbohydrates were extracted from samples that were composed of four shoots grown in the same culture container; five samples were collected per treatment and species, amounting to five biological replicates. Each replicate was adjusted to about 100 mg of fresh matter and ground in a 2 mL Eppendorf tube (automatic grinder for 1 min at 22 Hz) with two metallic beads and 1 mL of a water/ethanol mixture (20/80, v/v). After vortexing and shaking for 30 min at 4 °C with an Eppendorf Thermomixer at 1400 rpm, samples were centrifuged at 17,000× *g* at 4 °C for 10 min. The supernatant was collected, and the pellet extracted again with 0.5 mL water/ethanol (20/80, v/v). The resulting supernatant was pooled with the first and dried at reduced pressure (Speedvac). The final dried extract was re-suspended in 1 mL Milli-Q water and filtered at 0.45 μm (PVDF filters) prior to analysis using High Performance Anion Exchange Chromatography coupled to a Pulsed Amperometric Detector (HPAEC-PAD) (Dionex ED 40, Dionex Corp., Sunnyvale, CA, USA) [27]. The analytical column was a Dionex CarboPAC PA-20 (3 mm × 150 mm) kept at 35 °C. The mobile phase was an on-line generated KOH-gradient at a flow rate of 0.5 mL min^{-1}. The PAD detection was achieved with a gold working electrode and an Ag/AgCl reference electrode, with a data collection rate of 2 Hz. Carbohydrates were quantified using seven-points calibration curves with custom-made external standard solutions (based on stock solutions of arabinose, galactose, glucose, sucrose, xylose, fructose, melibiose, raffinose, stachyose, maltose, cellobiose and rhamnose), ranging 1–100 μmol L^{-1}. Every ten injections, one check standard solution was used to confirm the calibration of the system.

The concentration of sugars in the plant tissues was calculated based on the molar concentration obtained from the measurements and the initially fresh weight used. Data were analyzed using the one-way ANOVA with the least significant difference at $p > 0.05$, using the XLStat software.

Matrices of correlation (Pearson's correlation coefficients (n)) were calculated to compare the relation among the morphological parameters, the plant recovery and the concentrations of the sugars. Significance of values was tested with a level of alpha = 0.05 and p-value <0.05 using the XLSTAT software. Also, to interpret and visualize the correlations among the studied variables principal component analysis (PCA) was performed.

2.4. Preparation of protein extracts

Proteins were extracted from samples that were composed of eight shoots grown in the same culture container; five samples were taken per treatment and per species, amounting to five biological replicates.

For the extraction of proteins, shoots were ground in liquid nitrogen and mixed with 10 mL 20% *w*/*v* trichloroacetic acid (TCA) and 0.1% *w*/*v* dithiothreitol (DTT) in ice-cold acetone and kept overnight at −20 °C. After centrifugation for 45 min at 30,000× *g* and 4 °C, the pellets were washed twice with ice-cold acetone before drying. Dried samples were re-suspended in labeling buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 30 mM Tris) and incubated for 1 h at room temperature. After centrifugation (15,000× *g*, 15 min), the supernatant was transferred to 1.5 mL tubes.

Prior to quantification by Bradford method [10] with bovine serum albumin (BSA) as standard, the pH of the protein extracts was adjusted to 8.5.

2.5. Fluorescence labeling of proteins and two-dimensional gel electrophoresis

The extracts of proteins were used for analysis by 2-D fluorescence difference in-gel electrophoresis (2D-DIGE) [63].

The samples, four biological replicates, were homogenized by vortexing and centrifuged. Prior to electrophoresis, all protein extracts and a pooled internal standard were labeled with CyDyes™ (GE Healthcare, Little Chalfont, Buckinghamshire, UK) [40]. A volume, equivalent to 30 μg of protein, of each protein sample, was labeled with 240 pmol of the dyes. Two samples were pooled with 30 μg of the internal standard (amounting to 90 μg, 2 samples of 30 μg each and 30 μg of internal standard), the volume of this mixture adjusted to 120 μL with lysis buffer (labeling buffer without Tris and with bromophenol blue) and 2.4 μL of Destreak Reagent (GE Healthcare) and 0.72 μL of IPG buffer 4–7 NL (GE Healthcare) were added. This sample was then loaded by cup loading on 24 cm 4–7 ReadyStrip™ IPG strips (BioRad Laboratories, Inc, Hercules, CA, USA). Prior to the cup loading, the ReadyStrip™ IPG strips were rehydrated overnight with 450 μL of rehydration solution (prepared with 500 μL of Destreak rehydration solution (GE Healthcare) and 5 μL of IPG buffer pH 4–7 (GE Healthcare) [46].

Isoelectric focusing (IEF) was carried out on an Ettan IPGphor Manifold (GE Healthcare) in an IPGphor unit (GE Healthcare) with the following protocol: 150 V for 3 h, 300 V for 3 h, gradient to 1000 V over 6 h, gradient to 10,000 V over 3 h, 10,000 V for 8 h, 10,000 V until ~85,000 V h were reached at 20 °C with a maximum current setting of 50 μA per strip. After equilibration, reduction and alkylation of cysteines using DTT and IAA respectively, the strips were placed on 2D-Large-Gel flatbed NF 12.5% acrylamide pre-cast gels (Serva Electrophoresis, Heidelberg, Germany). The SDS-PAGE step was performed on High-Performance Electrophoresis (HPE)

FlatTop Tower (Serva Electrophoresis).

2.6. Image capture and analysis

The gel images of the samples were acquired using a Typhoon Variable Mode Imager 9400 (GE Healthcare) at a resolution of 100 μm according to the instructions provided for each dye. Images were analyzed using the Decyder v7.1 software (GE Healthcare). A two-way ANOVA with species as one factor and treatment as the second factor was performed. All spots with a significant score for one of the factors or the interaction between the two factors (p-value <0.05) were considered as spots of interest. A total of 347 spots were submitted to MS-based identification. The ratio of the spot intensities under the diverse conditions was determined. Moreover, for easy comparison the fold change, as given in the Supplementary data S4, the ratio was calculated using the following formulae; when A/B > 1 the fold change = A/B, when A/B < 1 then the fold change = −1/(A/B).

2.7. Protein identification

Spots of interest, selected as described earlier, were excised from a non-charged gel containing 90 μg of protein and digested using the fully automated Ettan Spot Handling Workstation (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as described previously [7]. All mass spectrometry (MS) and MS/MS analyses were done using a 5800 MALDI TOF/TOF (Applied Biosystems, Foster City, CA, USA) and the resulting spectra used in database searches with ProteinPilot (Applied Biosystems) and an in-house MASCOT platform (version 2.3.02, Matrix Science, www.matrixscience.com, London, UK). All proteins from the taxonomy Viridiplantae were downloaded from the NCBI server on the 18th of January 2013 and used as protein database; likewise the used expressed sequence tag (EST) database contained all ESTs from the taxon Solanaceae downloaded on the 29th of July 2013 (containing 1,162,105 and 7,908,438 sequences respectively). All searches (combined MS and 8 MS/MS spectra) were carried out using a mass window of 100 ppm for the precursor and 0.75 Da for the fragments. For the different searches, the following parameters were taken: two missed cleavages, fixed carbamidomethylation of cysteine and variable oxidation of methionine and tryptophan to kynurenine or double oxidation to N-formyl kynurenine. Complete identification data are given in the Supplementary data S4. When a protein was identified as "hypothetical", "unknown" or based on an EST sequence, the sequence was used for basic local alignment search tool (BLAST) and the protein with the highest homology (if significant) was added as identified protein. All identifications were manually validated, and extra precursors were selected for fragmentation if the obtained data were judged insufficient. When high-quality spectra were not matched to sequences, the sequence was determined manually, and/or the spectra were used for searches allowing for semitryptic peptides and common post-translational modifications (PTMs). This method resulted in the identification of several signal/transit peptide cleavage sites that were confirmed either by homology to known signal cleavage sites or by using predictive software (see Supplementary data S4B). For some spots, peptide sequences were determined manually, and the corresponding protein identified using MS-specific BLAST tools, as previously described [62]. Finally, protein hits based solely on peptide mass fingerprinting (PMF) were not accepted and in general only those proteins to which two non-identical peptides matched with scores above the MASCOT-determined threshold of 0.05 were considered identified. When a single MS/MS spectrum resulted in a match giving an expected value of below 10−5 for the protein and no extra sequence information could be obtained, the protein was considered identified after manual validation of the matched sequence. The functional classification of the identified proteins was done based on the gene ontology (GO) annotation from Goanna: [http://agbase.msstate.edu/cgi-bin/generateBlastPage.pl]).

3. Results and discussion

3.1. Morphological state of donor plantlets before cryoprocedure

Both stress treatments, sucrose and cold, had a significant negative effect on the increase of shoot length and the number of leaves in all four genotypes when compared to their controls (Table 1). The number of leaves was significantly fewer in cold cultured plants of *S. juzepcukii* 'Piñaza' and *S. commersonii* than in sucrose-pretreated plants. Cold also had a more distinct negative impact than the sucrose pretreatment on the increase of shoot length in precultured plantlets of *S. juzepcukii* 'Piñaza'. Likewise, the water content was significantly reduced by both pretreatments except for *S. ajanhuiri* 'Wila Yari' where only sucrose pretreatment reduced the water content (Table 1).

3.2. Cryopreservation results

Plant regeneration of non-frozen shoot tips was about 80–85% and neither preculture- nor genotype-dependent (data not shown). It was observed that post-rewarming meristem growth was lower (60–90%) compared to survival after cryopreservation (90–100%) (Data not shown). The meristem growth of rewarmed shoot tips from donor plants of *S. tuberosum* 'Désirée' was negatively affected by the cold pretreatment when compared to shoot tips from donor plants precultured under control and sucrose pretreatment media (Data not shown). Plant recovery (0–70%) of cryopreserved shoot tips exhibited a clear, pronounced preculture and genotype effect (Fig. 1). Sucrose pretreatment had a marked recovery effect, except for Wila Yari where no effect of any of the two pretreatments was observed. Only in *S. commersonii* had the cold pretreatment a positive impact on plant recovery. In previous experiments, it was found that rewarmed meristems from precultured donor plants of *S. commersonii* and *S. tuberosum* 'Désirée' showed high rates of survival and even meristem growth [25]. Our current results

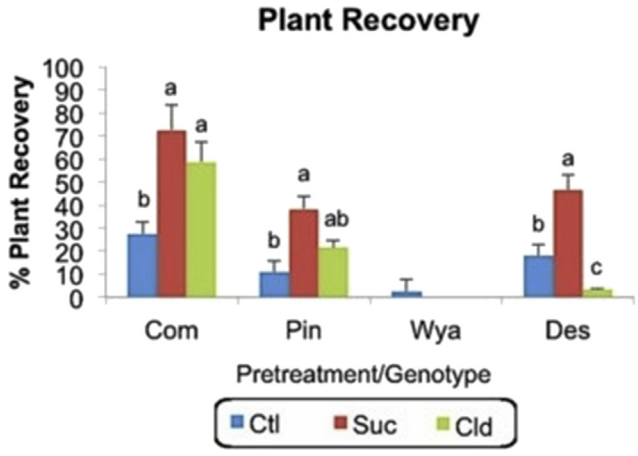

Fig. 1. Post cryopreservation results for the four potato genotypes [*S. commersonii* (Com), *S. juzepcukii* 'Piñaza' (Pin), *S. ajanhuiri* 'Wila Yari' (Wya) and *S. tuberosum* 'Désirée' (Des)], 28 days after rewarming from LN exposure. Prior to cryopreservation, donor plants were submitted to three treatments for 2 weeks; control (Ctl), sucrose (Suc) and cold (Cld).
*Different letters per parameter and genotype indicate values significantly different at $p \leq 0.05$ (ANOVA test). Error bars represent standard deviation.

indicate that plant recovery needs to be tested, since the majority of the cryopreserved shoot tips grew after rewarming, but they did not always results in a regenerated plant.

Cold preculture of shoot-tip donor plants is known to improve cryopreservation results especially in those species that acclimate to cold [61] and the type of cold acclimation may have different effects on the plant recovery [12,13]. In the current study, cold preculture enhanced plant recovery in meristems from donor plants of *S. commersonii* only (known to be frost-tolerant), but had no significant effect in *S. juzepcukii* 'Piñaza' (Fig. 1). Plant recovery results of meristems from cold precultured plants of *S. tuberosum* 'Désirée' were lower than those from control-cultured plants. In some plant species, cold acclimation can be replaced by sucrose incubation [17] or sucrose preculture [43]. Indeed, sucrose was used to preculture potato shoot tips prior to cryopreservation resulting in the acquisition of tolerance to cryopreservation [9,22,25,28,32,41]. Halmagyi et al. [28] obtained an increased percentage of shoot development after rewarming of meristems from *S. tuberosum* when they were pretreated with 0.5 M of sucrose for one day. For successful cryopreservation, it is essential to induce high levels of dehydration tolerance in plant cells, tissues and organs that are subjected to cryopreservation [66]. The vitrification solution, PVS2, also contains a high sucrose concentration that causes, together with the other compounds, desiccation of tissues prior to cryopreservation. These concentrated sugar solutions are also known to be good glass formers and stabilizers of labile macromolecules when aqueous solutions are subjected to cryogenic temperatures [44]. This procedure helped to obtain a high percentage of survival after cryopreservation in all the studied conditions independent of the preculture of donor plants. An improvement in plant recovery was obtained by applying the sucrose pretreatment medium in donor plants of *S. commersonii*, *S. juzepcukii* 'Piñaza' and *S. tuberosum* 'Désirée' in comparison to the control precultures (Fig. 1). However, neither preculture resulted in any improvement of cryopreservation results in *S. ajanhuiri* 'Wila Yari'. The reduction in growth and the water loss might prepare the plantlets to better face the cryopreservation protocol. However, these parameters alone are not sufficient to describe the distinct plant recovery yields, since all genotypes have a similar behavior in plant growth and water status when subjected to the same culture conditions. This effect suggests that the response to the cryoprocedure is not only influenced by preculture conditions but is also genotype-dependent. Amongst others, this might be linked to a differential production of reactive oxygen species (ROS) during the cryoprocedure, that may be accumulated and produce cellular damage. A number of recent studies show that the addition of antioxidants during the cryopreservation process could reduce oxidative damage and can thus result in improved regrowth of both non-frozen and cryopreserved plant tissues [15,69,70,74]. This study aimed to test the effect of pretreatments before cryopreservation on the plant regeneration. No additives, such as growth regulators or antioxidants, were added to the recovery medium. Therefore, it is only the ability of the shoot tip (acquired during the preculture and/or given by each genotype) that affects the tolerance to cryopreservation and partially explains the distinct plant regeneration rates. However, the high viability of the shoot tips after cryopreservation indicates there is a margin of improvement for the cryoprocedure by optimizing the recovery conditions after rewarming.

3.3. Changes in soluble sugars after preculture and their relationship to cryopreservation tolerance

Results from previous experiments have shown changes in the content of soluble sugars in plants exposed to osmotic and cold stress treatments [24,25]. Sugar concentrations can alter dramatically in response to environmental signals such as abiotic stress [6], and they are known to be involved in cryopreservation ability.

In the current experiment, the content of nine soluble sugars (glucose, fructose, sucrose, rhamnose, arabinose, xylose, galactose, raffinose and stachyose) was measured. It was observed that the accumulation of soluble sugars is preculture- and/or genotype-dependent. Except for xylose, the concentrations changed significantly in most plantlets subjected to stress conditions when compared to control (Fig. 2).

Matrices of correlation were calculated (as described in material and methods) to verify whether there is a link between plant morphology, changes in sugar concentration and plant recovery (Table 1). Furthermore, a PCA was performed to visualize the samples based on the correlation among the variables. The distribution of the observation maps on the PCA plots shows that the differences among samples are both genotype and treatment-dependent (Fig. 3). The observation maps of each species also demonstrate the treatment-induced differences (Supplementary data S1A-D). Stressed plants of *S. commersonii* and *S. tuberosum* 'Désirée' respond similarly indicating that the response to stress is similar in both species. The samples of *S. ajanhuiri* 'Wila Yari' of cold-cultured and control plants group together and are separated according the F1 axis from sucrose precultured plants. This distribution shows that the cold pretreatment has less effect on the parameters included in the test than the sucrose pretreatment (Supplementary data S1D).

Sucrose accumulates significantly in donor plants of *S. commersonii* and *S. juzepcukii* 'Piñaza' subjected to sucrose pretreatment medium as well as in cold-cultured plants of *S. commersonii* (Fig. 2). Plant cells take up sucrose and after culture on sucrose pretreatment medium, alterations of biomembranes, such as the presence of multi-membranous structures and the undulations of the plasma membrane caused by partial plasmolysis were observed [29]. Helliot et al. [29] showed other ultrastructural changes like the fragmentation of vacuoles due to sucrose preculture of proliferating meristems. In a previous study, accumulation of sucrose was found to be appropriate for cryopreservation ability and response to stress conditions in sucrose-treated and cold-cultured potato plants (*S. commersonii* and *S. tuberosum* 'Désirée') [25]. In the current work, involving more potato genotypes, a significant positive correlation ($r = 0.39$) of sucrose concentration in donor plants with plant recovery after cryopreservation was obtained. Moreover the concentrations of sucrose was negatively correlated with morphological parameters, such as increase in shoot length ($r = -0.45$), number of leaves ($r = -0.38$) and water content ($r = -0.68$) (Supplementary data S2A.1). These results indicate that the sucrose concentration in precultured donor plants might not only be relevant for response to stress but also for plant recovery after rewarming of the meristems that are excised from sucrose-treated donor plants. However, when considered per accession, the correlation coefficient of sucrose concentration was not significantly correlated with plant recovery in any of the genotypes (Supplementary data S2A.2-5). A significant negative correlation between sucrose and increase of shoot length ($r = -0.80$), number of leaves ($r = -0.84$) or water content ($r = -0.77$) was only found in plants of *S. commersonii* (Supplementary data S2A.2).

When taking all genotypes together, a significant positive correlation ($r = 0.71$) was found for plant recovery of meristems from cold-cultured donor plants and the sucrose concentration in donor plants (Supplementary Data S2B.3). Such correlation was not found after sucrose preculture (Supplementary Data S2B.2) indicating a possible saturation of sucrose in the cell's cytoplasm. Studies on cryopreservation of banana meristems suggested that the sucrose

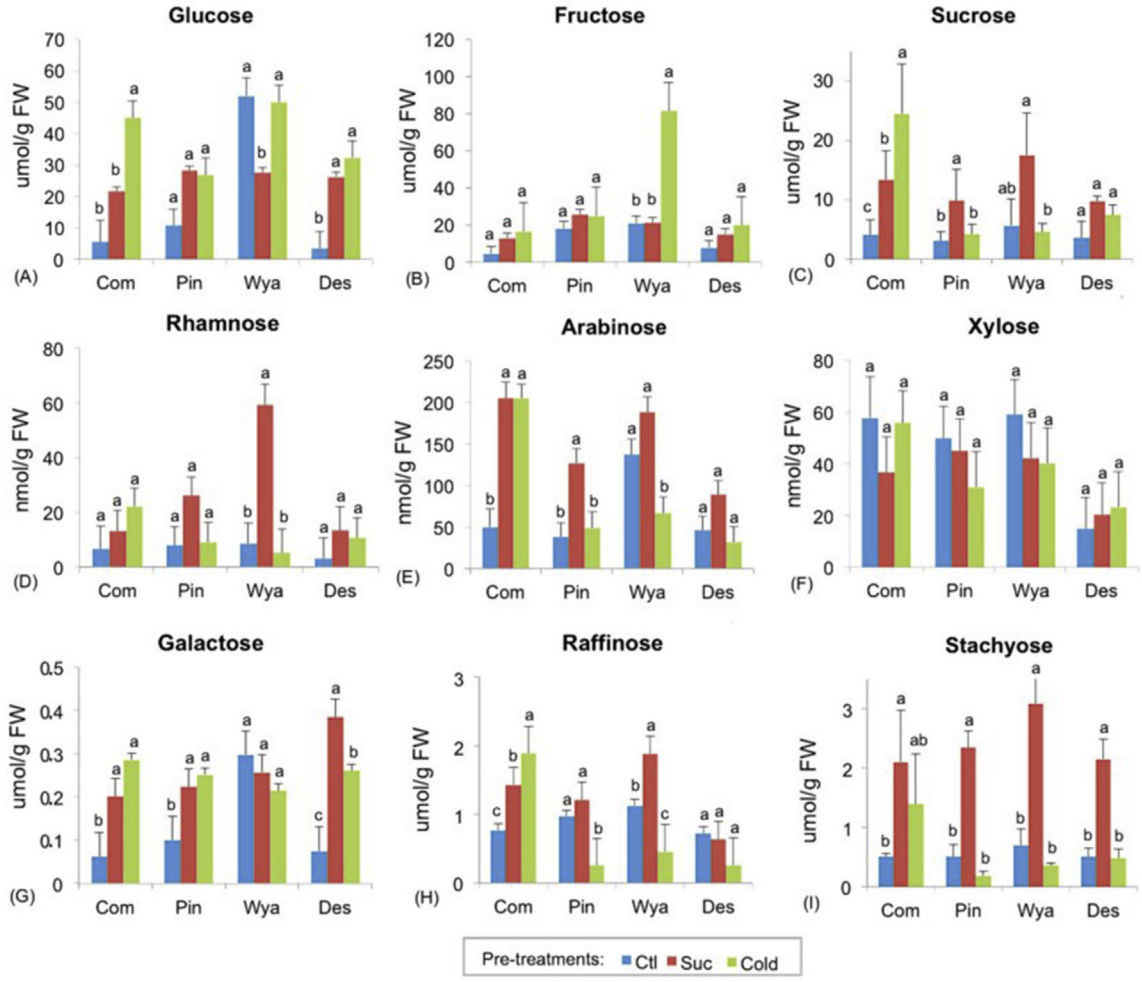

Fig. 2. Concentration of (A) glucose, (B) fructose, (C) sucrose, (D) rhamnose, (E) arabinose, (F) xylose, (G) galactose, (H) raffinose and (I) stachyose in potato shoot tips that were submitted to different treatments for 2 weeks (Ctl, control; Suc, sucrose; Cld, Cold). Statistical significance was tested by one-way ANOVA among pretreatments within each genotype [*S. commersonii* (Com), *S. juzepcukii* 'Piñaza' (Pin), *S. ajanhuiri* 'Wila Yari' (Wya) and *S. tuberosum* 'Désirée' (Des)].
*Different letters per parameter and genotype indicate values significantly different at $p \leq 0.05$ (ANOVA test). Error bars represent standard deviation.

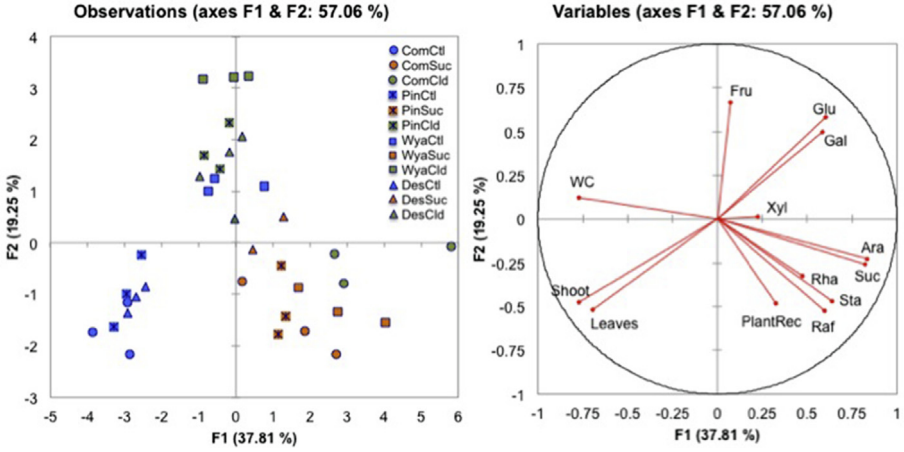

Fig. 3. PCA score plots of four genotypes [*S. commersonii* (Com), circles; *S. juzepcukii* 'Piñaza' (Pin), squares with cross; *S. ajanhuiri* 'Wila Yari' (Wya), squares; *S. tuberosum* 'Désirée' (Des), triangles] analyzed together for all preculture conditions (Ctl, control in blue; Suc, sucrose in orange; Cld, cold in green): it is based on morphological parameters, plant recovery and soluble sugars. The graph at the left side represents observation groups and the graph at the right side represents correlation scores of each variable (Fru, fructose; Glu, glucose; Gal, galactose; Xyl, xylose; Ara, arabinose; Suc, sucrose; Rha, rhamnose; Sta, stachyose; Raf, raffinose; PlantRec, plant recovery after cryopreservation; Leaves, number of leaves of donor plants; Shoot, increase of shoot length in donor plants; Water content (WC) in donor plants).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the total sugar content are the main determining factors for successful cryopreservation [76]. Our results indicate that the sucrose and the associated WC might be limiting factors after cold pretreatment. The lack of correlation between sucrose content in donor plants treated with sucrose pretreatment medium and the regrowth of meristems from sucrose-cultured donor plants suggests that there are other limiting factors influencing the recovery in this case. Zhu et al. [76] found that sucrose-induced changes of membrane components, such as sterols and fatty acids are linked to the cryopreservation ability of banana meristems.

In this study, besides alterations in sucrose contents, changes in the concentration of other sugars that might be relevant for cryopreservation tolerance were observed (Fig. 2). During drought stress not only sucrose, but also other soluble carbohydrates, particularly those from the raffinose family oligosaccharides (RFOs, such as raffinose, stachyose, verbascose), can contribute to osmotic adjustments as well as to membrane and protein stabilization [1,68]. RFOs were found to accumulate under drought stress and help to stabilize cell proteins [4], and/or act as antioxidants to counteract the accumulation of reactive oxygen species (ROS) under stress conditions [8,39,50,64,71,72].

Our results showed an accumulation of RFOs, particularly stachyose, in donor plantlets under osmotic stress conditions (sucrose-enriched medium). Raffinose accumulates in *S. commersonii* and *S. ajanhuiri* 'Wila Yari' (Fig. 2H–I). Galactose accumulates after sucrose pretreatment in three accessions (Fig. 2G). It was proposed that all these sugars contribute towards subcellular protection against desiccation injury to facilitate recovery on rehydration [55]. The correlation between plant recovery and raffinose or stachyose concentrations is not significant (though positive) if all accessions are considered (Supplementary Data S2A.1). However, a significant positive correlation of stachyose concentration with plant recovery is observed for *S. commersonii* ($r = 0.77$), *S. juzepcukii* 'Piñaza' ($r = 0.71$) and *S. tuberosum* 'Désirée' ($r = 0.78$) when considered individually (Supplementary Data S2A.2,3,5). Therefore, it is hypothesized that the impact of the accumulation of raffinose and stachyose on plant recovery is genotype dependent. Besides, significant positive correlations of plant recovery with the accumulation of raffinose ($r = 0.85$) and stachyose ($r = 0.61$) were shown in cold-cultured plants (Supplementary Data S2B.3). The concentration of sucrose is furthermore positively correlated with raffinose ($r = 0.88$) and stachyose ($r = 0.94$) concentrations in cold-cultured plants, suggesting an influence of the preculture conditions (Supplementary Data S2B.3). These results suggest that these three sugars could contribute to the lower water content observed under cold conditions and that their accumulation has a positive effect on plant recovery. A significant accumulation of raffinose in plantlets of *S. commersonii* subjected to cold pretreatment was observed (Fig. 2H). Raffinose was shown to accumulate in several species during cold acclimation [2,11,33,65]. Precisely *S. commersonii* is considered one of the most frost-tolerant potatoes [14], whereas the other three genotypes in this study have diverse levels of sensitivity to cold [31]. Various studies have suggested that raffinose can act as antioxidant/osmoprotectant thus increasing the tolerance to oxidative stress [48,50]. The results of the current study indicate that the raffinose might have a cold acclimation effect on *S. commersonii*, and the accumulation of raffinose might help to acquire tolerance to cryopreservation. Raffinose was used as a cryoprotectant in cryopreservation of sperm [67], oocytes [20] and chloroplast membranes [45].

Meristems from donor plants of *S. ajanhuiri* 'Wila Yari' precultured on sucrose-enriched medium, which significantly accumulated raffinose and stachyose, did not show an improved plant recovery after rewarming. Though the survival and meristem growth percentages in *S. ajanhuiri* 'Wila Yari' were high (60–90%), plant recovery was around zero. These results showed that while the water content could be linked to the accumulation of sugars like sucrose, raffinose or stachyose in the other genotypes, no significant correlation was found for *S. ajanhuiri* 'Wila Yari' (Supplementary Data S2A). Hence, there must be other crucial factors that are involved, such as *in vitro* regeneration capacities [5]. The meristems of *S. ajanhuiri* 'Wila Yari' only start growing three weeks after rewarming, which is about one week later than the other genotypes. Moreover,a follow-up of the plant recovery for more than four weeks confirmed they were not able to regrow into a plant. These results indicate that there are other parameters, besides those studied in the current work, that affect the plant recovery of *S. ajanhuiri* 'Wila Yari'. In this case, the focus should be laid on the meristem culture and regeneration capacity.

3.4. Proteome changes of precultured plants

The effects of the two preculture treatments on the proteome of the plantlets were tested with a two-way ANOVA, with genotype as factor one and treatment as factor two. Differentially abundant proteins with a fold change of <-1.5 or >1.5 and a $p < 0.05$ were selected. This method resulted in the selection of 381 spots that were significantly distinct. In 315 of these spots, at least one protein could be significantly identified (Supplementary data S4). Elimination of the spots in which two or more proteins had been identified resulted in a final list of 141 differentially abundant spots, containing only one identified protein that can be considered for biological interpretation and discussion (Table 2, Fig. 4 and Supplementary data S4B). Spots containing the same protein or homologous proteins with the same function were grouped resulting in a summary of 65 protein families, classified by function (Table 2).

It was observed that *S. commersonii* shows a relatively uniform response under the two stress conditions; half of the proteins that change in abundance are common in plants that were cultured on sucrose pretreatment medium and cold. However, the two stress conditions induced more dissimilar changes in the proteomes of the three other genotypes (Table 2).

Carbon metabolism-associated proteins are the main class that is affected in sucrose- and cold-exposed plants (23%–69% of the total differentially abundant proteins) (Table 2). In previous studies, changes in abundance of carbon metabolism-related proteins in potato plants submitted to osmotic and cold stress were described [24] and they were related to the regulation of metabolic processes such as photosynthesis [25]. However, the changes in abundance of carbon metabolism-related proteins are similar among the four genotypes. For the plants of *S. juzepcukii* 'Piñaza' and *S. ajanhuiri* 'Wila Yari', the changes in abundance depend on the particular stress treatment (*S. juzepcukii* 'Piñaza' is more affected by cold while *S. ajanhuiri* 'Wila Yari' is more affected by sucrose).

In previous studies, some stress-related proteins (such as proteinase inhibitors, heat-shock proteins and proteins associated with the maintenance of the ROS-homeostasis) changed in abundance when potato plantlets were exposed to sucrose and cold stress conditions [24,25]. These proteins are involved in a general response to stress and particularly in osmotic and chilling stress in potato. In the current study also changes in abundance of some proteins involved in the response to stress and oxidative homeostasis were observed, the most frequently identified spots being proteinase inhibitors and heat-shock proteins (Table 2). The oxidative-homeostasis system is affected by the pretreatments and might also affect the tolerance to cryopreservation.

The results show an increased accumulation of proteinase inhibitors in plants of *S. juzepcukii* 'Piñaza', and *S. tuberosum* 'Désirée' when exposed to sucrose or cold pretreatment. However, the

Table 2
Summary of proteins that changed in abundance arranged by functional classification.

Biological process[a]	Number of spots[b]															
	Sucrose/Control[c]								Cold/Control[d]							
	Com[e]		Pin[f]		Wya[g]		Des[h]		Com		Pin		Wya		Des	
Total	**4**	**47**	**8**	**4**	**20**	**11**	**12**	**15**	**8**	**44**	**14**	**21**	**8**	**14**	**11**	**7**
Carbon metabolism	1	33	2	2	8	7	4	8	3	32	2	11	3	0	4	5
Protein metabolism	2	1	3	0	0	0	1	1	0	1	5	0	2	0	2	0
Transcription and translation	0	1	0	1	1	2	1	0	0	1	0	0	0	1	0	1
Lipid metabolism	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Oxidative homeostasis	0	3	0	0	4	1	1	0	0	4	0	1	0	2	1	0
Response to stress	0	4	2	0	2	1	4	3	1	1	7	1	3	6	4	0
Other cellular processes	1	4	1	1	2	0	1	3	2	4	0	6	0	5	0	0
Not assigned	0	1	0	0	3	0	0	0	0	1	0	2	0	0	0	1

[a]Biological process: name of the biological processes in which identified proteins are involved, based in their functional classification; [b]Number of spots: number of spots containing one identified protein, which significantly increases (green) or decreases (red) in abundance (in grey if there is no significant varying protein); [c]Sucrose/Control: comparison between Sucrose and Control conditions in each genotype; [d]Cold/Control: comparison between Cold and Control conditions in each genotype; [e]Com: *S. commersonii*; [f]Pin: *S. juzepcukii* 'Piñaza' (Pin); [g]Wya: *S. ajanhuiri* 'Wila Yari'; [h]Des: *S. tuberosum* 'Désirée'. Numbers in bold represent the total number of spots that significantly change in abundance.

Tables with detailed information on the identification data and the fold changes of the individual spots are available as Supplementary Data S3.

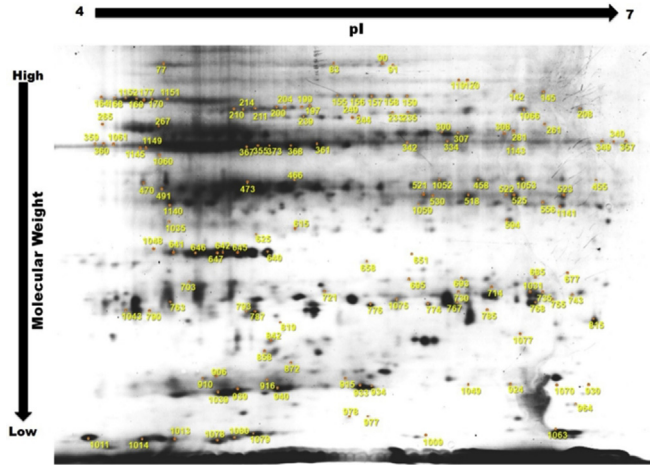

Fig. 4. Two-dimensional electrophoresis potato shoot proteome gel map. The master gel is shown with the 141 identified spots considered for the biological interpretation. Isoelectric point range (pI) is represented from the left to the right and the molecular weight from upper to bottom part of the picture.

response of *S. ajanhuiri* 'Wila Yari' varies depending on the pre-treatment; two spots containing a proteinase inhibitor were found to increase in abundance under sucrose pretreatment while six spots decrease in abundance in cold-cultured plants. A treatment-dependent effect was also found for *S. commersonii*; none of these proteinase inhibitors changed in abundance in cold-cultured plants while one proteinase inhibitor decreased in abundance in plants of *S. commersonii* exposed to sucrose. Proteinase inhibitors were reported in several studies on abiotic stress responses [24,26,42,46,60] and they were suggested to play a major role in the tolerance to stress and developmental processes of members of the Solanaceae family [30]. Other stress-related proteins such as heat shock protein 70 (Hsp70) were identified in several spots. Particularly Hsp70 decreased in abundance in plants of *S. commersonii* and *S. tuberosum* 'Désirée' when cultured on sucrose-enriched medium while they accumulated in all cold-cultured plants. While none of the spots containing Hsp70 changed in abundance in *S. juzepcukii* 'Piñaza' or *S. ajanhuiri* 'Wila Yari' when they were exposed to sucrose pretreatment, several spots wherein Hsp70 was identified accumulated or decreased in abundance in plantlets of both accessions exposed to cold. Hsp70 is one of the Hsps that were proposed as a marker of plant stress [73]. These results suggest that the group of Hsp proteins might play a role in the response to abiotic stress in potato as they are implicated in regulatory mechanisms of the cells [47]. However, further studies are still needed to confirm the role these stress-related proteins might play in the tolerance to the cryopreservation procedure.

In summary, this work shows that the response of donor plants to stress conditions can be related to the acquisition of tolerance to cryopreservation by the excised meristems. The reduction in growth and the water loss might prepare the plantlets to face the cryopreservation protocol. Our results suggest that oxidative homeostasis-related proteins and sugars are associated with the improved tolerance to cryopreservation and the ability to cold acclimate by *S. commersonii* in contrast to the other genotypes. These results also support the idea that sucrose and RFOs play a key role in the stress response of potato, and the accumulation of these sugars may help to acquire tolerance to the cryopreservation procedure. Based on the results here, we propose the preculture of donor plantlets on sucrose pretreatment medium. As raffinose was used as a cryoprotectant, a future venue for research should, therefore, study whether cryopreservation results can be further improved by adding RFOs to the preculture medium in potato. Besides, further investigations are still needed to unravel the effect of recovery media on the plant regeneration.

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Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cryobiol.2015.09.006.

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