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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).

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