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Original article

Does pre-culture in sugar-rich media affect carbohydrate content and post-thawing recovery rate of cryopreserved potato (*Solanum phureja*) shoot tips?

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ABSTRACT

Shoot tips of Potato (*Solanum phureja*) were cryopreserved with the encapsulation-dehydration method, including: coating of shoot tips in calcium alginate beads, pre-culture in a solution containing 0.75 M of sugar (0.1 M of sucrose +0.65 M of another cryoprotectant sugar), and dehydration (for 4.5 h) on silica gel to reduce water content to 21–27%. Regrowth of shoot tips was assessed after freezing in liquid nitrogen. After cryopreservation, coated shoot tips were cultivated on a medium enriched with growth substances to measure concentrations of soluble carbohydrates. Maltose and trehalose ensured a better cryopreservation (88% and 91% of regrowth, respectively). Regrowth rates of potato shoot tips in the presence of polyols were very low and no recovery was obtained after freezing of shoot tips that were pre-cultivated in monosaccharides. Concentration of soluble carbohydrates increased in shoot tips pre-cultivated in sucrose, trehalose or glucose, especially when sucrose was applied as the cryoprotectant. The application of sucrose helped potato shoot-tips to acquire a certain tolerance to dehydration and cryofreezing. The analysis of soluble carbohydrates in potato shoot tips confirmed the accumulation of sugars during the pre-culture process, principally in the form of sucrose. The statistical analysis showed that soluble carbohydrate contents in shoot tips increased when total carbohydrates increased in the cultivation medium. The modelling approach indicated that the contents of soluble carbohydrates increased when pre-cultivation of potato shoot-tips was conducted in sucrose media compared to the control and other sugars.

1. Introduction

In vitro culture was developed as a tool for the conservation of a wide range of plant species. Large scale propagation, somatic embryogenesis, and production of metabolites are other important fields of application (Bouafia et al., 1995; Mehalaine and Chenchouni, 2020). Nevertheless, the use of *in vitro* culture methods for long-term conservation can induce physiological, genetic and epigenetic changes (Muthusamy et al., 2007; Yang and Ye, 2013). Other limitations of *in vitro* culture conservation are high maintenance cost, risk of contamination and mixture of genotypes. The preservation at very low temperatures "cryopreservation", at −196 °C, is currently the sole technique that allows safe long-term preservation of biological materials (Engelmann, 2004). The low temperature of liquid nitrogen stops cell metabolism and can thus ensure a long-term preservation of pathogen-free genotypes without the risk of genetic drifts.

Several studies tried to identify the main factors that induce variations in regrowth of cryopreserved shoot tips of cultivated varieties and wild species of potato (Engelmann, 2004; Kim et al., 2006; Kaczmarczyk et al., 2008; Kaczmarczyk et al., 2011). Pre-

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culture, cultivation conditions of shoot tip donor plants, cooling, warming and post-culture treatments were identified as crucial factors for successful cryopreservation (Kaczmarczyk et al., 2011). Already cultivated potato varieties and wild potato species were successfully cryopreserved and showed high regrowth rates after thawing (Kim et al., 2006). However, the mode of action of cryoprotectants is poorly known, probably due to the presence of various complex mechanisms. Cryoprotectants with low molecular weight, generally penetrate into the cells and act in a colligative way, *i.e.* proportionally to the number of molecules per unit of water volume (Arakawa et al., 1990). Other cryoprotectant agents have a more specific action and are called non-colligative cryoprotectants, forming preferentially hydrogen bonds with membrane proteins and phospholipids (Rodrigues et al., 2008).

Potato is the basic food ingredient in most daily dishes of many countries worldwide, including Algeria (Oustani et al., 2015). Thus, achieving mass production of this crop and improving agriculture production in terms of quantity and quality is a national strategy in Algeria, which is adapted also by several developing countries (Fellah et al., 2018; Mihi et al., 2019). The modernization of agriculture depends on the improvement of scientific research in agricultural and agri-food sectors. The modernization of potato production system includes the application of techniques and tools for seed preparation using cryo-preservation protocols of plant genetic resources, as well as the use of appropriate methods of cultivation for planting and even best practices of irrigation and harvesting (Fellah et al., 2018; Boudjabi et al., 2015, 2019). So in order to achieve food sovereignty, a revolution in agricultural production systems, including potato cropping, must be rethought in this context. The present study aims at improving the recovery rates of cryopreserved potato shoot tips (*Solanum phureja*) using different cryoprotectant molecules. The effect of adding cryoprotectant agents to the pre-culture media was assessed with a set of eleven cryoprotectants. Total and soluble carbohydrate contents in shoot tips were determined using High-Performance Liquid Chromatography (HPLC) following Black et al. (1996), in order to better understand the mechanisms of sucrose action during dehydration and cryopreservation (Fabre and Dereuddre, 1987; Dumet, 1994; Folgado et al., 2015). Hereafter, total carbohydrates or total sugars refer to the sum of the sugars that were analysed.

2. Materials and methods

2.1. In vitro culture

In vitro potato plants (*Solanum phureja*) were obtained from the GERMICOPA SA company (France). Mono-nodal stem cuttings of 1 cm of length were excised from 6 to 8 week-old *in vitro* potato plants on fresh "cutting" medium. The culture medium contained macroelements of Tendille and Lecerf (1974) i.e. (808.8 mg/L KNO_3, 160.1 mg/L NH_4NO_3, 141.7 mg/L $Ca(NO_3)_2.4\ H_2O$, 123.3 mg/L $MgSO_4{\cdot}7H_2O$, 104.4 mg/L KCl, 81.7 mg/L KH_2PO_4, 7.0 mg/L K_2HPO_4), microelements of Murashige and Skoog (1962): (6. 2 mg/L H_3BO_3, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}22.3$ mg/L $4H_2O$, 0.83 mg/L KI, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 8.6 mg/L $ZnSO_4{\cdot}7H_2O$, 5.57 g/L $FeSO_4{\cdot}7H_2O$, 7.45 g/L Na_2-EDTA, 0.025 mg/L $CuSO_4{\cdot}5H_2O$), vitamins of Morel and Wetmore (1951): (100 mg/L inositol, 1 mg/L thiamine.HCl, 1 mg/L nicotinic acid, 1 mg/L pyridoxine.HCl, 1 mg/L Ca pantothenate, 0.01 mg/L biotin, Na-Fe-EDTA, 30 g/L sucrose, 8 g/L agar, pH 5.8), 30 g/L of sucrose, and 8 g/L of Difco® Bacto agar. The pH was adjusted to 5.85 with NaOH (1 N) and HCl (1 N). Agar was added at a temperature of 100 °C, followed by autoclaving at 110 °C for 20 min.

2.2. Shoot tip excision

For propagation of shoot tip donor plants, mono-nodal stem cuttings were sub-cultured for seven days in deep petri dishes (Ø = 10 cm) and incubated under the above described conditions, under room temperature (20 ± 1 °C), relative humidity = 70 ± 5%, photoperiod = 16 h, and light intensity = 50 μmoles/m^2/s. Potato shoot tips of a length of 0.5–1.0 mm (with few leaf primordia) were excised under the stereoscope.

2.3. Encapsulation procedures

Excised potato shoot tips were placed in liquid Ca-free medium, containing macroelements (500 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 125 mg/L $MgSO_4\ 7H_2O$, 1000 mg/L KCl, 125 mg/L KH_2PO_4, 1000 mg/L $(NH_4)_2$-SO_4) of Morel and Muller (1964), microelements of Heller (1953) i.e. (0.1 mg/L $MnSO_4{\cdot}4H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 1 mg/L H_3BO_3, 0.01 mg/L KI, 0.03 mg/L $CuSO_4{\cdot}5H_2O$, 0.03 mg/L $AlCl_3$, 0.03 mg/L $NiCl_2{\cdot}6H_2O$, 1 mg/L $FeCl_3{\cdot}6H_2O$), vitamins of Morel and Wetmore (1951), with EDTA-$FeNa_2$, 5 mg/L of gibberellic acid (GA_3), 0.001 mg/L of 1-naphtaleneacetic acid (NAA), 0.01 mg/L 6-benzylaminopurine (BAP), 34.2 g/L of sucrose (0.1 M), supplemented with 30 g/L of sodium alginate (3%). For encapsulation, shoot tips and solution were absorbed with a micropipette, and dropped in an alginate-free culture medium (same composition), supplemented with 14.7 g/L of calcium chloride ($CaCl_2$, $2H_2O$). Grown beads have a diameter of 3–4 mm and contained 1–2 shoot tips per bead. Beads were rinsed twice with liquid Ca-free shoot tip medium and pre-cultured on semi-solid shoot tip medium, supplemented with different types of cryoprotectants (sugars and polyols).

2.4. Cryoprotection (pre-culture)

A standard concentration of 0.1 M of sucrose was maintained for the encapsulation and pre-culture media. A final osmolarity of 0.75 M was obtained, adding the equivalent of 0.65 M of each cryoprotectant to the pre-culture media (Bouafia et al., 1995). Eleven different cryoprotective pre-culture treatments were assessed: 0.1 M of sucrose plus 0.65 M of different monosaccharides (glucose, fructose and galactose), disaccharides (trehalose, cellobiose, maltose and lactose), triholoside (raffinose), mannitol, sorbitol and sucrose. Coated shoot tips were pre-cultivated for two days under the previously described conditions (see subsection: *2.1. In vitro culture*).

2.5. Dehydration on silica gel and freezing in liquid nitrogen (LN)

Pre-cultured shoot tips were dehydrated for 4.5 h on silica gel at a temperature of 20 °C. Shoot tips' water content was reduced to 0.20–0.22 g of water/g DM. Shoot tips were transferred into cryobiological tubes, directly plunged in LN and maintained for minimum 1 h in LN. Water content of beads [in %] was calculated as (fresh weight−dry weight)/fresh weight, which represents weight loss.

2.6. Rewarming and plant recovery

Shoot tips were rewarmed at room temperature (~20 °C), directly placed on shoot tip medium, and incubated at 20 ± 1 °C and a relative humidity of 70 ± 5%, with a photoperiod of 16 h and light intensity of 50 μmoles/m^2/s, provided by True lite Durolux fluorescent tubes.

2.7. Determination of total and soluble carbohydrates in shoot tips

Total carbohydrate content was measured in both untreated (taken directly from the mono-nodal stem cuttings) and coated shoot tips. Shoot tips were pre-cultured for 2 days on shoot tip culture media, supplemented with 0.65 M of trehalose, glucose and sucrose (total osmolarity = 0.75 M). Pre-culture conditions were the same as previously described (see subsection: *2.1. In vitro culture*). After pre-culture, shoot tips were extracted from the coating using a scalpel and rinsed with demineralized water. Shoot tips were fixed in 0.5 mL of ethanol (96% vol.) and stored at −25 °C. The experiment was repeated three times with a sample size of 20–25 shoot tips per treatment.

Shoot tips were crushed in the presence of 1 mL of ethanol (96% vol.) and centrifuged at 2000g for 10 min (at 4 °C). The supernatant was collected (ethanolic extract), the pellet was resuspended in 1 mL of water, crushed and centrifuged again to obtain the aqueous extract. Supernatants were stored at −25 °C after that the ethanolic and aqueous extracts were vacuum evaporated for 2–5 h. The dry extract was diluted in 200 μL of distilled water. Charged molecules were removed from the extract solution using an ion exchange resin mini-column. Samples of 20 μL of extract solution were analysed using high performance liquid chromatography (HPLC). Quantitative measurement was carried out with the integration method. Two reference solutions were used, the first contained sucrose, glucose and fructose, and the second enclosed the same carbohydrates plus trehalose.

2.8. Data analysis

Variation in fresh (FMW) and dry weight matter (DMW) of empty beads as well as FMW/DMW ratio and weight loss in % (FMW-DMW/FMW) was assessed among a set of 11 cryoprotectants using one-way analyses of variance (ANOVA) followed by Tukey's post hoc tests. Normal distribution of data points was tested using the Shapiro test. The effect of the cryoprotectant molecular mass on weight loss and fresh and dry weights of encapsulated potato shoot tips (in beads) was analysed using a generalized linear model (GLM), including Gaussian error family and identity link. Raw data used in computations is available online at: https://dx.doi.org/10.6084/m9.figshare.4542454.

The effect of dehydration (-LN), liquid nitrogen exposure (+LN) and pre-culture with different cryoprotectants on the shoot tips' recovery rate was analysed with a two-way ANOVA test. Separated ANOVA tests were performed for two cryoprotectant categories (disaccharides, trisaccharides and polyols). Variations of concentrations of total soluble carbohydrates and individual sugars in potato shoot tips, pre-cultured on three different cryoprotectants (sucrose, trehalose and glucose), were analysed with Tukey's HSD test. The variation of different soluble carbohydrate concentrations following total carbohydrates of different pre-culture treatments was tested using GLM with Gaussian distribution error and identity link. Statistical analysis was carried out using R software (R Core Team, 2019).

3. Results

3.1. Fresh and dry matter weights of pre-cultured beads

Pre-cultured empty beads (without shoot tips) were used to determine if the cryoprotectant treatment altered the water retention potential of the beads. After pre-culture in liquid monosaccharide-rich culture media (glucose, fructose and galactose), empty calcium alginate beads showed DMWs ranging from 145.5 to 158 mg. FWM and DMW of empty alginate beads increased significantly after pre-culture in disaccharide-rich culture media. Beads pre-cultured in sucrose- and trehalose-rich media had FMWs of 439.3 ± 3.3 mg and 266.6 ± 2.1 mg, respectively. Beads pre-cultured in maltose-, cellobiose- and lactose-rich culture media showed FMWs of 282.0 ± 2.4 mg, 336.9 ± 3.2 mg, and 335.2 ± 3.0 mg, respectively (Table 1). The highest DMWs were observed when beads were pre-cultured in culture media containing raffinose (318.6 ± 2.6 mg) or sucrose (345.6 ± 2.8 mg). Low DMWs were observed in beads pre-cultured in mannitol- (138.6 ± 2.1 mg) and sorbitol-rich (150.1 ± 0.2 mg) culture media.

The ANOVA test showed significant differences for DMWs, FMWs, FMW/DMW ratio and weight loss between the assessed cryoprotectants ($P < 0.001$). Following Tukey test, the lowest values of FMW/DMW ratio were recorded with sucrose (1.271) and raffinose (1.27) whereas the highest scores were observed with fructose (1.366) and lactose (1.353). Regarding the weight loss, it was the lowest in mannitol and trehalose with an average of 21%, and the highest in fructose, galactose and cellobiose with about 26% (Table 1).

The used General Linear Model showed a significant positive relationship between changes in FMWs and DMWs of empty alginate beads and the molecular mass of the respective cryoprotectant. The use of cryoprotectants with heavy molecular mass induced a significant increase in FMW (GLM: $F = 88.4$, $P < 0.001$) and DMW (GLM: $F = 93.0$, $P < 0.001$) of the pre-cultivated empty beads (Fig. 1). However, weight loss decreased significantly as

Table 1
Fresh (FMW), dry matter weight (DMW), FMW/DMW ratio, and weight loss in % ((FMW-DMW)/FMW) of empty beads, pre-cultured for two days in liquid culture media supplemented with 11 different cryoprotectants. MM: molecular mass. The pre-culture media contained 0.1 M of sucrose and 0.65 M of the respective cryoprotectant. Values are expressed as means ± standard deviations (SD). *F* (*F*-statistics) and *P*-value summarize one-way analyses of variance (ANOVAs). Different letters indicate significant differences for empty bead traits between cryoprotectants using Tukey's post-hoc test ($P < 0.01$).

Cryoprotectants	MM [g/mol]	FMW [mg]	DMW [mg]	FMW/DMW ratio	Weight loss [%]
Glucose	180	207.2 ± 0.8^{c}	158.0 ± 0.2^{c}	1.311 ± 0.004^{d}	23.55 ± 0.39 ^{bc}
Fructose	180	214.8 ± 1.2^{d}	157.3 ± 0.4^{c}	1.366 ± 0.011^{e}	26.78 ± 0.58 ^{a}
Galactose	180	198.1 ± 1.8^{b}	145.5 ± 0.6^{b}	1.361 ± 0.015^{e}	26.54 ± 0.78 ^{a}
Maltose	342	282.0 ± 2.4^{f}	221.0 ± 2.4 ^{e}	1.276 ± 0.003^{ab}	23.73 ± 0.21^{b}
Cellobiose	342	336.9 ± 3.2^{g}	257.5 ± 3.4 ^{g}	1.308 ± 0.007^{cd}	26.1 ± 0.33 ^{a}
Sucrose	342	439.3 ± 3.3^{i}	345.6 ± 2.8 ^{i}	1.271 ± 0.002^{a}	21.63 ± 0.19 ^{de}
Lactose	342	335.2 ± 3.0^{g}	247.7 ± 2.1^{f}	1.353 ± 0.006^{e}	23.26 ± 0.08 ^{bc}
Trehalose	342	266.6 ± 2.1^{e}	206.3 ± 2.1 ^{d}	1.292 ± 0.003^{bc}	21.26 ± 0.21 ^{e}
Raffinose	504	404.6 ± 2.2 ^{h}	318.6 ± 2.6 ^{h}	1.270 ± 0.003^{a}	22.97 ± 0.14 ^{bc}
Mannitol	182	180.6 ± 2.5^{a}	138.6 ± 2.1 ^{a}	1.303 ± 0.001^{cd}	21.33 ± 0.13 ^{e}
Sorbitol	182	194.9 ± 0.6^{b}	150.1 ± 0.2^{b}	1.298 ± 0.002^{cd}	22.61 ± 0.16 ^{cd}
Overall		278.2 ± 87.0	213.3 ± 70.0	1.310 ± 0.035	23.61 ± 1.98
$F_{(10, 22)}$		4659	3817	88.9	96.71
P-value		<0.0001	<0.0001	<0.0001	<0.0001

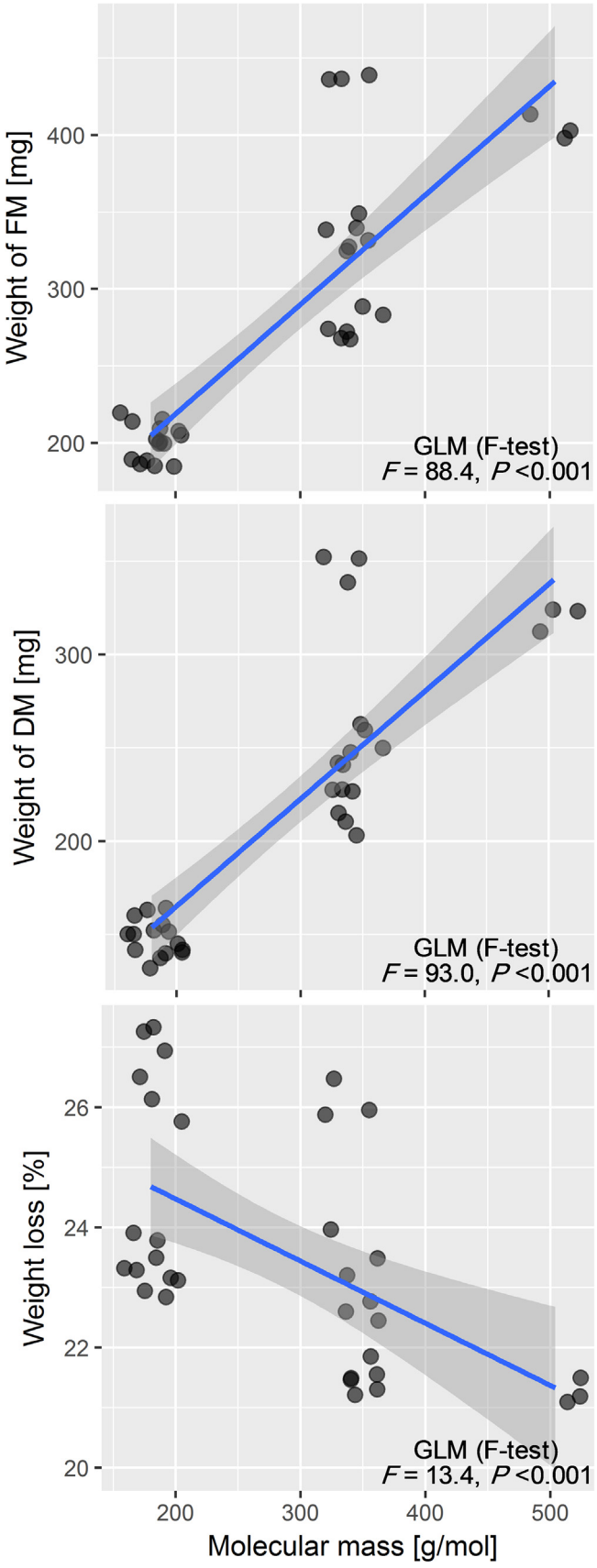

Fig. 1. Relationship between the molecular mass of cryoprotectants and weigh loss, weights of fresh (FM) and dry matter (DM) of empty beads of potato. The lines represent a linear regression with a GLM fit (generalized linear model) and 95% confidence region in light grey. *F* (*F*-statistics) and *P* (*P*-value) are results of GLMs (Gaussian distribution error and 'Identity' link).

the molecular mass of cryoprotectants increased (GLM: $F = 13.4$, $P < 0.001$).

3.2. Tolerance to pre-culture, dehydration and freezing

Coated pre-cultured shoot tips showed an average recovery rates (rr) of 53% without liquid nitrogen exposure (-LN) and average 39% for in liquid nitrogen frozen samples (+LN). Dehydration with mannitol showed the highest recovery rate of 100% (-LN). No regrowth was observed when shoot tips were dehydrated in monosaccharide-rich culture medium (water content of beads: 21–27%). The pre-culture of shoot tips in disaccharide-rich culture media considerably increased its tolerance to dehydration (-LN) and freezing in liquid nitrogen (+LN). Highest post-thawing recovery rates were observed for shoot tips pre-cultured in culture media containing trehalose (rr = 91%), maltose (rr = 88%), and sucrose (rr = 78%). The ANOVA test showed significant variations for disaccharide cryoprotectants and treatments (Table 2). Shoot tips pre-cultured in culture media supplemented with polyols showed lower recovery rates after LN exposure. The ANOVA showed significant differences for the recovery rates of shoot tips pre-cultured in trisaccharide- and polyol-rich culture media ($P < 0.001$) (Fig. 2, Table 2).

3.3. Composition of shoot tips with soluble carbohydrates

The ANOVA showed significant differences for the total carbohydrate content of potato shoot tips pre-cultured in culture media containing three different types of cryoprotectants ($F_{(3,\ 32)} = 2556$, $P < 0.001$). For the control treatment (without pre-culture), shoot tips showed a total carbohydrate content of 33 ± 1.2 mg/g FMW. The highest carbohydrate content was observed when shoot tips were pre-cultured in trehalose-rich medium (Fig. 3). Contents of total sugars (i.e. sum of the sugars that were analysed) in controls averaged 14.6 mg/g FMW of sucrose, 9.7 mg/g FMW of glucose and 8.7 mg/g FMW of fructose (Fig. 4). After pre-culture in 0.75 M sucrose-rich medium, the total soluble carbohydrate content was 48.4 ± 1.6 mg/g FMW (1.46 times higher than that for the control treatment). The total concentration of carbohydrates (78.3 ± 0.8 mg/g FMW) significantly increased after pre-culture in trehalose-rich culture medium. This increase was related to the high sucrose content (57.5 mg/g FMW), representing 73.4% of the total carbohydrates of shoot tips. The contents of glucose (14%) and fructose (12.6%) constituted only minor portions of the total carbohydrate content. For the glucose-rich pre-culture medium, shoot tips showed a total carbohydrate content of 58.4 ± 0.7 mg/g FMW. Glucose represented 33% (19.3 mg/g FMW), whereas sucrose was dominant with 50.5% of total sugars (29.5 mg/g FM) (Fig. 3, Fig. 4). Total soluble carbohydrate content was significantly different for pre-culture treatments ($P < 0.004$) and type of soluble carbohydrates ($P < 0.001$). The composition of the soluble carbohydrates (glucose, fructose and sucrose content) varied significantly following the cryoprotectant molecule used in pre-culture. The variation of soluble carbohydrate contents was higher in sucrose and glucose ($P < 0.001$) compared to fructose ($P = 0.035$) (Table 3).

3.4. Relationships between rich-media total carbohydrates and soluble carbohydrates in shoot tips

The GLM testing the variation of different soluble carbohydrate concentrations following total carbohydrates and pre-culture treatments showed that soluble carbohydrate contents increased when total carbohydrates increased in the media. However, this relationship was not significant (GLM: $t = 0.71$, $P = 0.491$) (Table 4). The model indicated also that the contents of soluble carbohy-

Table 2
Analysis of Variance (ANOVA) of the recovery rates of encapsulated potato shoot tips. Coated potato shoot tips were pre-cultured for 2 days in liquid culture media supplemented with 11 different types of cryoprotectants, followed by freezing in liquid nitrogen (+LN) or direct recovery without liquid nitrogen exposure (-LN) (treatment factor). ANOVA test was performed separately for disaccharides, trisaccharides and polyols together, and all cryoprotectants. *DF*: Degrees of Freedom; *SS*: Sum of Squares; *MS*: Mean Squares, *F*: *F*-statistics, *P*: *P*-value.

Variables	*DF*	*SS*	*MS*	*F*	*P*
All cryoprotectants					
Treatment	1	3462	3462	2218	<0.001
Cryoprotectants	10	87,153	8715	5585	<0.001
Treatment × Cryoprotectants	10	10,811	1081	693	<0.001
Residuals	44	69	2		
Disaccharides					
Treatment	1	496	496	266	<0.001
Cryoprotectants	4	2427	607	325	<0.001
Treatment × Cryoprotectants	4	182	46	24	<0.001
Residuals	20	37	2		
Trisaccharides + polyols					
Treatment	1	7041	7041	2697	<0.001
Cryoprotectants	2	4379	2189	838	<0.001
Treatment × Cryoprotectants	2	6553	3277	1255	<0.001
Residuals	12	31	3		

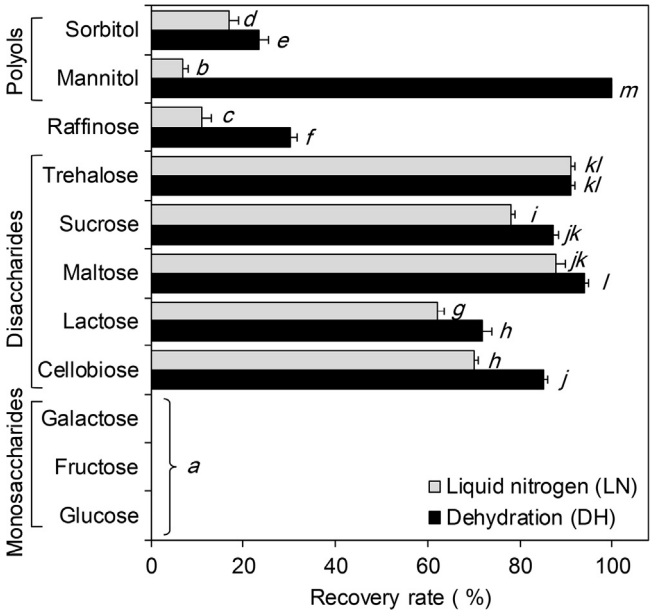

Fig. 2. Recovery rates of shoot tips pre-cultivated in various cryoprotectant-based culture medium after dehydration and freezing in liquid nitrogen. Italic letters indicate results of Tukey's HSD tests, where the same letter are not significantly different at $P > 0.05$.

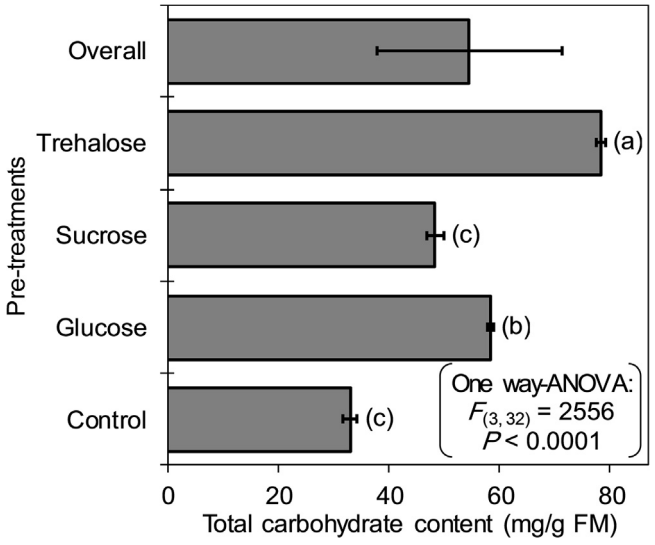

Fig. 3. Contents of total carbohydrates measured in potato shoot tips directly pre-cultivated in three cryoprotectants "sucrose, trehalose and glucose" (n = 3, with each replicate composed of 20–25 shoots). Error bars represent standard deviations. The control included shoot tips not pre-cultivated. Letters between brackets indicate results of Tukey's HSD tests, where the same letter are not significantly different at $P > 0.05$.

drates increased when the pre-cultivation of potato shoot-tips was carried out in sucrose compared to control and other sugars (Fig. 5). The GLM also revealed that regardless of total carbohydrate concentrations in pre-culture treatments, no differences ($P > 0.05$) were observed in concentrations of the soluble carbohydrates in shoot-tips (Table 4).

4. Discussion

Regrowth of coated shoot tips of potato (*Solanum phureja*) after cryopreservation is strongly influenced by the pre-culture step. When shoot tips were pre-cultured for two days in a solution containing 0.75 M of sucrose, a post-thawing recovery rate of 78% was obtained (+LN). Natural tolerance to dehydration of some organisms is related to the modification of metabolism, including metabolism of sugars (Sun et al., 1994; Pinker et al., 2009; Folgado et al., 2015), as well as the accumulation of sucrose (Quain et al., 2009). Pre-culture of potato (*Solanum tuberosum)* shoot tips in sucrose-rich culture medium had a positive effect on plant regeneration (Folgado et al., 2014; Folgado et al., 2015). The penetration of sucrose into cells during the pre-culture stage can be explained by the accumulation of starch in the shoot tips of potato (Fabre, 1991) and date palm (Bagniol, 1992; Bagniol et al., 1992), but also in cell suspensions of *Catharantus roseus* (Bachiri, 1994). Pre-culture with sugar increases the shoot tips' tolerance to dehydration and freezing in LN (Bouafia et al., 1996). Folgado et al. (2015) reported that pre-culture conditions improved plant recovery in the *Solanum juzepcukii* species.

The efficacy of sucrose can be questioned when it is replaced by other cryoprotectants. Following a pre-culture with trehalose or maltose at a concentration of 0.65 M, the respective recovery rates of 91 and 88% (+LN) were higher than those obtained with sucrose (78%). Other disaccharides showed to be less effective, with recovery rates of 70% (cellobiose) and 60% (lactose). Shoot tips pre-cultivated on trehalose-rich culture medium (0.65 M of trehalose plus 0.1 M of sucrose) showed a significantly higher content of

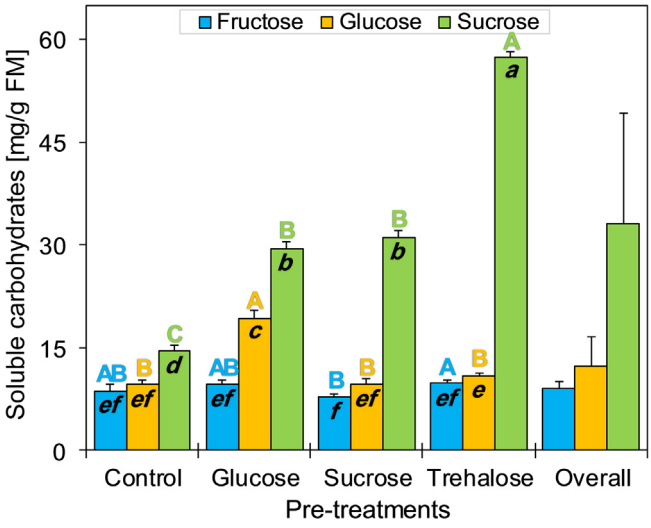

Fig. 4. Variation of soluble carbohydrate contents in potato shoot tips pre-cultivated based on the application of various cryoprotectants "sucrose, trehalose and glucose" (n = 3, with each replicate composed of 20–25 shoots). The control included un-precultivated shoot tips. Vertical bars represent standard deviations. Italic lowercase letters in black colour indicate results of Tukey's HSD tests associated with two-way ANOVA (Table 3), where the same letter are not significantly different at $P > 0.05$. Capital and coloured letters (top of bars) are Tukey's HSD tests associated with one-way ANOVAs (comparisons of each soluble carbohydrate between pretreatments).

total sugars, which is principally due to an increase in trehalose and sucrose content (Fig. 3). A similar process was observed in buds of grapevines (Plessis, 1994) and eucalyptus (Monod, 1995).

In the current study, monosaccharides were demonstrated to be completely ineffective for the cryopreservation of potato shoot tips with the encapsulation-dehydration method. Similar results were reported by Bouafia et al. (1996). The relatively high water content in beads (21–27%) may be the main cause for the poor tolerance of potato shoot tips to cryopreservation. Nevertheless, in previous studies glucose was reported as a good cryoprotectant (Green and Angell, 1989). The cryoprotective effectiveness of glucose was tested on somatic embryos of carrot (Tessereau. 1993), oil palm (Dumet, 1994), chinaberry (Scocchi et al., 2007), maritime pine (Alvarez et al., 2012), as well on shoot tips of carnation (Fabre and Dereuddre, 1987), grapevine (Plessis, 1994), on membranes of thylakoids isolated from spinach (Santarius, 1996), and on potato (Bouafia et al., 1996). The tolerance of potato shoot tips to freezing in LN was negative when pre-culture was done in polyol-rich culture media. This may be explained by the low solubility of sorbitol, mannitol and raffinose. Indeed, beads pre-cultivated in mannitol-rich medium lost their translucency and become white during the dehydration process. Similar results were reported in earlier studies (Dumet, 1994; Monod, 1995). Pre-culture under cold conditions negatively affected the recovery rate of some potato species (Folgado et al., 2015).

As a cryoprotectant, sucrose plays a determining role in the acquisition of tolerance to dehydration and freezing in potato

Table 3
ANOVAs testing the variation of soluble carbohydrate contents in potato shoot tips after two days of pre-culture in liquid culture media containing three different types of cryoprotectants (sucrose, trehalose and glucose). Sugar contents were determined using High Performance Liquid Chromatography (HPLC). *DF*: Degrees of Freedom; *SS*: Sum of Squares; *MS*: Mean Squares, *F*: *F*-statistics, *P*: *P*-value.

Variables	*DF*	*SS*	*MS*	*F*	*P*
All soluble carbohydrates (two-way ANOVA)					
Pre-culture	3	1080.5	360.2	5.4	0.004
Type of carbohydrate	2	4104.4	2052.2	30.9	<0.001
Residuals	30	1995.2	66.5		
Glucose contents (one-way ANOVA)					
Pre-culture	3	194.4	64.8	101.5	<0.001
Residuals	8	5.1	0.6		
Fructose contents (one-way ANOVA)					
Pre-culture	3	7.1	2.4	4.7	0.035
Residuals	8	4.0	0.5		
Sucrose contents (one-way ANOVA)					
Pre-culture	3	2859.3	953.1	1311.6	<0.001
Residuals	8	5.8	0.7		

Table 4
Parameters of the generalized linear model (GLM) testing the relationships between total carbohydrate content and different soluble carbohydrate concentrations for various pre-culture treatments of cryopreserved potato shoot tips.

Variables	Estimate	2.5% CI	97.5% CI	SE	*t*-value	*P*-value
Intercept	−0.47	−25.75	24.81	12.90	−0.04	0.972
Soluble carbohydrate = Glucose 'SCG'	18.90	−16.85	54.66	18.24	1.04	0.321
Soluble carbohydrate = Sucrose 'SCS'	−1.42	−37.17	34.33	18.24	−0.08	0.939
Total carbohydrate content 'TCC'	0.28	−0.49	1.04	0.39	0.71	0.491
Pre-culture in Glucose 'PCG'	−29.89	−116.56	56.79	44.22	−0.68	0.512
Pre-culture in Sucrose 'PCS'	0.94	−36.80	38.69	19.26	0.05	0.962
Pre-culture in Trehalose 'PCT'	−17.66	−114.12	78.80	49.22	−0.36	0.726
SCG × TCC	−0.54	−1.63	0.54	0.55	−0.99	0.343
SCS × TCC	0.22	−0.86	1.30	0.55	0.40	0.695
SCG × PCG	−16.89	−139.46	105.69	62.54	−0.27	0.792
SCS × PCG	−10.41	−132.98	112.17	62.54	−0.17	0.871
SCG × PCS	−28.33	−81.71	25.06	27.24	−1.04	0.319
SCS × PCS	55.26	1.88	108.64	27.24	2.03	0.065
SCG × PCT	−5.86	−142.28	130.56	69.60	−0.08	0.934
SCS × PCT	99.49	−36.93	235.91	69.60	1.43	0.178
TCC × PCG	0.41	−1.21	2.02	0.82	0.49	0.630
TCC × PCS	−0.12	−1.08	0.83	0.49	−0.26	0.803
TCC × PCT	0.08	−1.33	1.49	0.72	0.11	0.915

Table 4 (*continued*)

Variables	Estimate	2.5% CI	97.5% CI	SE	*t*-value	*P*-value
SCG × TCC × PCG	0.68	−1.60	2.96	1.16	0.58	0.572
SCS × TCC × PCG	0.32	−1.96	2.60	1.16	0.28	0.787
SCG × TCC × PCS	0.78	−0.58	2.13	0.69	1.12	0.283
SCS × TCC × PCS	−0.86	−2.21	0.50	0.69	−1.24	0.240
SCG × TCC × PCT	0.39	−1.61	2.39	1.02	0.39	0.706
SCS × TCC × PCT	−0.87	−2.86	1.13	1.02	−0.85	0.413

(2.5% CI and 97.5% CI: lower and upper Confidence Intervals, SE: Standard Error).

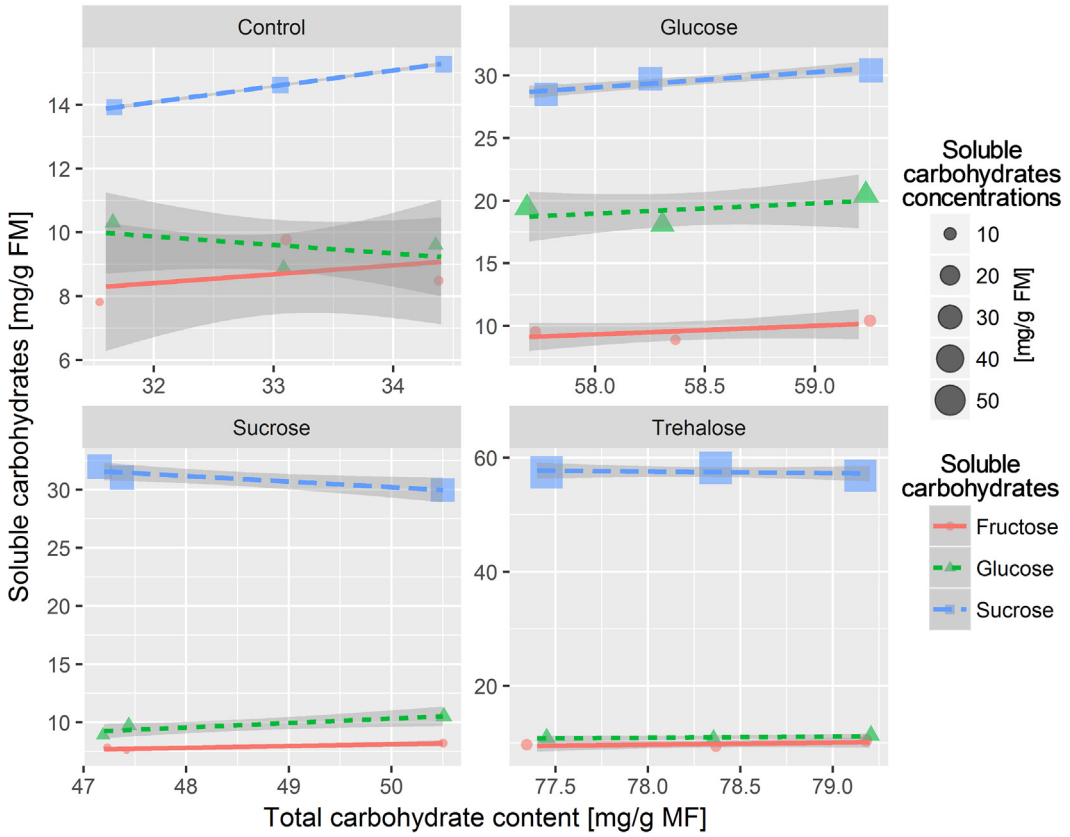

Fig. 5. Relationship between total carbohydrate content and different soluble carbohydrate concentrations for various pre-culture treatments of cryopreserved potato shoot tips. Values are set to soluble carbohydrate concentrations and the shape and colour of points represent the type of soluble carbohydrate. The coloured lines represent linear regressions obtained by a Gaussian GLM fit (generalized linear model) with confidence regions in light grey.

shoot tips. Other disaccharides like trehalose (recovery rate = 91%) and maltose (rr = 88%) showed inclusive to be effective as good as sucrose. The analysis of soluble carbohydrates in potato shoot tips confirmed the accumulation of sugars during the pre-culture process, principally in the form of sucrose, which increases recovery rates as the tolerance of cryopreserved plants to dehydration and cryofreezing improves compared to untreated plants.

5. Authors' contributions

Samia Bissati and Christiane Morisset designed the study. Samia Bissati carried our laboratory work. Samia Bissati, Saliha Boudjenah and Haroun Chenchouni drafted the manuscript. Haroun Chenchouni analysed data and revised the article. All authors read and approved the manuscript.

6. Availability of data and materials

The datasets used and/or analysed during the current study are available online at: https://dx.doi.org/10.6084/m9.figshare.4542454, or from the corresponding author on a reasonable request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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