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VITRIFICATION OF POTATO SHOOT TIPS FOR GERMPLASM CRYOPRESERVATION

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Abstract: Shoot tips of in vitro grown potato (*Solanum tuberosum* L.) plants were cryopreserved by a modified vitrification method. One-month-old potato plants were used for apices preparation. Successefully vitrified and warmed apices resumed growth within 4 weeks. For the evaluation of the regenerative capacity only directly developed shoots without intermediary callus formation were considered. The use of the PVS2 solution resulted in higher regeneration rates compared to the other mixtures tested (PVS1 and PVS3). Thus, in the case of a PVS2 treatment for 15 minutes, at a temperature of 0ºC, 42% of the shoot tips regenerated, and in the case of a treatment for 15 minutes at a temperature of 25ºC, 49% of the shoot tips regenerated shoots after cryopreservation.

Introduction

A successful cryopreservation, with a view to maintaining the viability of the plant material postfreezing, requires the knowledge of the processes that take place during freezing and warming, as well as of their effects on cellular biostructures. The duration and intensity of dehydration are relevant for the appearance of cellular damage during the freezing/warming cycle. Cellular damage depends on the cell water content, the elasticity of cell walls, the permeability of the plasmatic membrane, as well as on the freezing/warming rate [12]. A too marked dehydration of cells causes damage through the intracellular effect produced by the concentration of the endocellular solution that generates a toxicity of these compounds. As a consequence of this effect, protein denaturation occurs in a first stage, and if the situation persists, a process of oxidation of the SH groups is triggered [12]. Through the selection of an optimal freezing/warming regime and of adequate cryoprotective treatments this damage can be minimized.

Vitrification in the context of cryopreservation consists of the transition of a water solution into a solid, vitreous, amorphous mass, phenomenon which occurs at or below the freezing temperature of the water solution [5]. Theoretically, all cryoprotective agents currently used in cryopreservation vitrify at extremely low temperatures providing they are in sufficiently high concentrations in the mass to be frozen [8]. In order to vitrify the plant material – at the contact with liquid nitrogen – this should be treated with cryoprotective agents in extremely high concentrations. This treatment results in a concentration of the solutions in the cell, as a consequence of the penetration of the cryoprotective agents, which allows at -196°C the whole cell content to turn into an amorphous, vitreous mass.

Vitrification as a preservation method has two main characteristics: 1) the presence of extremely high cooling rates and 2) the use of extremely high concentrations of cryoprotective agents.

In addition to apices [14, 2] cryopreservation by vitrification has been performed in other types of inocula, such as: a) cell suspensions [1], b) protoplasts [3], c) somatic embryos [15] or d) transformed roots [17].

Materials and Methods

In vitro potato cultures (*Solanum tuberosum* L., cv. Désirée, Ostara, Santé) were initiated from the shoots grown on the potato tubercles and were multiplied by subcultures performed periodically on a MS [6] medium supplemented with GA3 0.2 mg/l, Z 1 mg/l, IAA 0.5 mg/l, glycine 1 mg/l and agar 7 g/l (=V_1). Thus, with a view to multiplication, the potato plants were shaped into binodal minicuttings which were inoculated on fresh nutritive media. The temperature in the growth chamber was 25±1°C and the photoperiod corresponded to 16 light hours/24 hours and a light intensity of 39.06 $\mu E.m^{-2}.s^{-1}$.

For cryopreservation apical shoot tips (3-4 mm in lenght) were used, which were excised using hypodermic needles and a microscop (x40) under aseptic conditions. After excision, the shoot tips were transferred to Petri dishes, on filter paper humidified with a liquid culture medium: MS + GA3 0.2 mg/l + Z 1 mg/l + IAA 0.5 mg/l + glycine 1 mg/l (=V_2), for 24 hours. Subsequently, they were incubated in various cryoprotective solutions for 10 minutes, after which they were frozen. The shoot tips were transferred onto aluminium foils (0.5/2.5 cm), in one drop (4 µl) of vitrification mixture (for 15 minutes), which were immersed in liquid nitrogen. For the vitrification of the cell content, various mixtures were used: PVS [15], b) PVS2 [10], c) PVS3 [7]. The shoot tips were warmed in liquid medium and in air at room temperature. The medium used for plant regeneration following cryopreservation was the medium V_1, which had a semisolid consistency, gelification being performed in this case with agar 3.5 g/l.

The percentage of plant regeneration after cryopreservation was estimated four weeks after warming and transfer of shoot tips to the regeneration media.

In order to assess the regenerative capacity, only direct plantlet regeneration was considered based on the fact that the most conclusive test to demonstrate the viability of shoot tips is their capacity to regenerate whole plants. The regenerative capacity was evaluated by relating the percentage of regenerated shoots to the total number of cryopreserved shoot tips.

Abbreviations: DMSO-dimethylsulfoxid, EG-ethylene glycol, GA3-gibberellic acid, IAA-indole-3-acetic acid, MS-Murashige and Skoog (1962), PEG-8000-polyethylene glycol, PVS2-vitrification solution, Z-zeatin.

Results and Discussion

Effect of cryoprotective substances on the regrowth of shoot tips

Due to the fact that the vitrification method eliminated the operation of gradual decrease in temperature, during which protective cellular dehydration is carried out, a different dehydration modality was required in compensation. In order to avoid osmotic shock, which could lead to cell death, this treatment was performed in two stages. In a first stage, the shoot tips were partially dehydrated as a result of their incubation in various cryoprotective mixtures (for 10 minutes), and in the second stage, they were exposed for 15 minutes in concentrated PVS2 solution.

The results regarding the influence of the various cryoprotective mixtures is presented in Fig. 1a, b, c.

Fig. 1a, b, c shows that the individual use of the cryoprotective substances DMSO and EG did not lead to plant regeneration from shoot tips, following cryopreservation in the case of cv. Ostara and cv. Santé (Fig. 1b, c), (variants 1 and 2), and it led to an insignificant regeneration rate of 7% in the case of cv. Désirée (Fig. 1a), (variant 2).

The highest regeneration rates in the case of cv. Désirée and cv. Ostara, 58% and 53%, respectively, were obtained with the combination PEG 10% + EG 1 M + DMSO 5% (variant 9), and in the case of cv. Santé 51%, with the cryoprotective mixture consisting of PEG 20% + EG 0.5 M + DMSO 10% (variant 10).

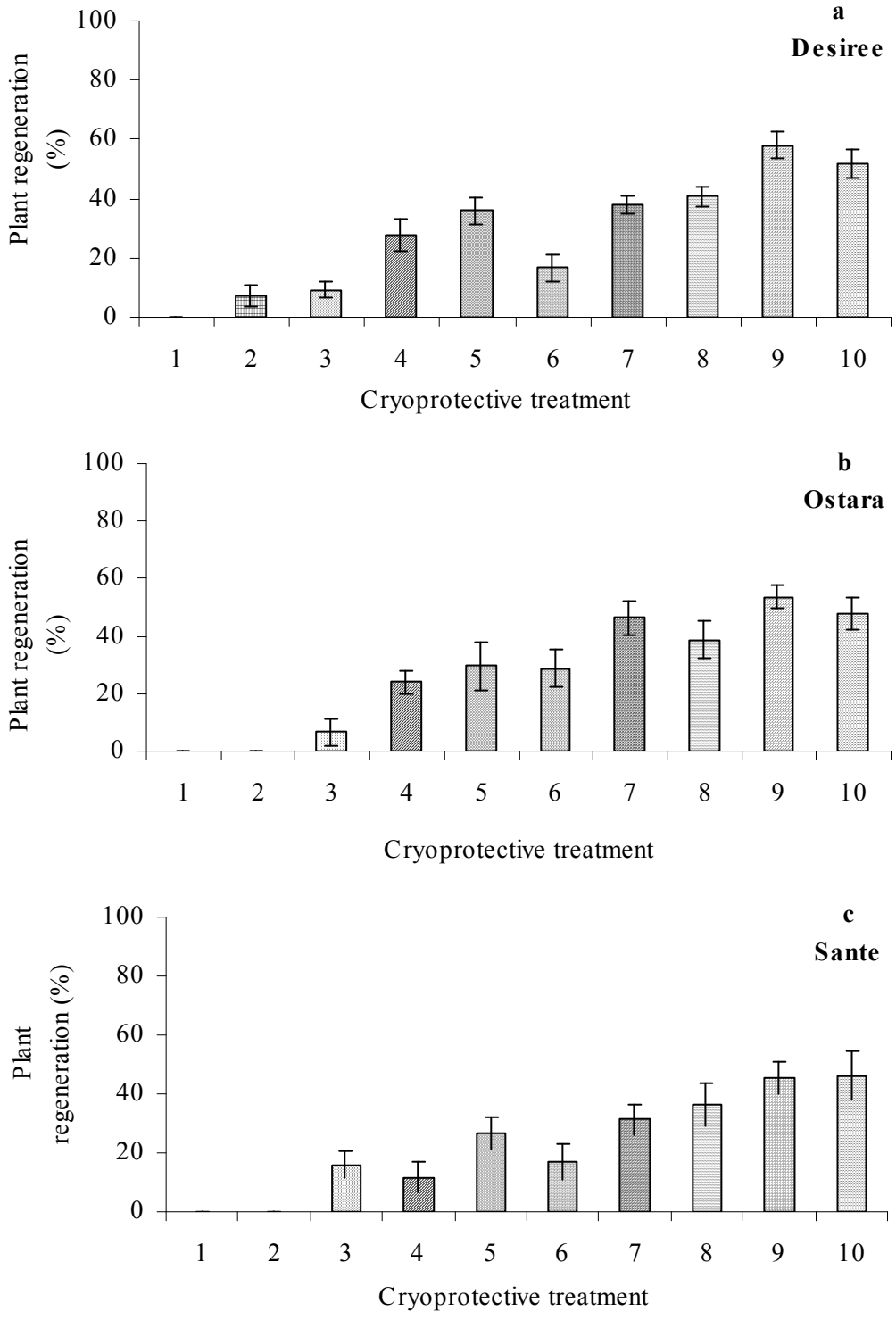

Fig. 1a, b, c: Effect of cryoprotective treatment on the plant development following shoot tips cryopreservation 1) DMSO 10%; 2) EG 10%; 3) PEG 10%; 4) Sucrose 0.4 M + DMSO 10%; 5) Sucrose 0.4 M + glycerol 0.5 M; 6) Sucrose 0.4 M + EG 0.5 M; 7) Sucrose 0.4 M + EG 1 M + DMSO 5%; 8) Sucrose 0.4 M + EG 0.5 M + DMSO 10%; 9) PEG 10% + EG 1 M + DMSO 5%; 10) PEG 20% + EG 0.5 M + DMSO 10%.

Langis and Steponkus [3] considered the duration necessary for the equilibration of concentrations between the intracellular and extracellular media to vary between 5 and 25 minutes, depending on the temperature at which this phase is carried out. Some authors [1, 9] used a single cryoprotective solution in both stages of application of the vitrifying mixture. There is no *a priori* reason for which the concentrated solution of cryoprotective mixture should be used in the first stage, on the contrary, there are several reasons for which it should be different [3, 4].

Effect of vitrification solutions on the regrowth of shoot tips

The following experiments used only cv. Ostara. After the cryoprotective treatment described above, the cell content was sufficiently concentrated to tolerate the exposure to cryoprotective solutions in increased concentrations for a more marked dehydration.

In the case of cryopreservation by vitrification, it is important to determine the toxicity of the cryoprotective mixture intended to ensure cellular vitrification, without endocellular crystallization, at the time of the immersion of tissues in liquid nitrogen, as well as to find the optimum time and temperature for the exposure of the plant material to these mixtures. In order to find the optimum cryoprotective mixture for the vitrification of potato shoot tips, the effect of three vitrification solutions was tested (Table 1).

Table 1: Influence of the composition of cryoprotective mixtures on the maintenance of the regenerative capacity of shoot tips (cv. Ostara) after cryopreservation

Cryoprotective mixtures	**Temperature** (ºC)	Duration (minutes)	Shoot regeneration (controls) (%)	Shoot regeneration (after cryopreservation) (%)
PVS	0ºC	5	84.3±4.04*	17.3±3.05
		15	81.6±6.42	24.0±5.29
	25ºC	5	81.3±4.16	28.0±3.60
		15	83.3±6.50	34.0±6.08
PVS2	0ºC	5	87.3±7.02	36.6±3.51
		15	91.0±2.64	42.0±5.29
	25ºC	5	84.3±5.13	34.0±4.58
		15	86.3±7.09	49.3±5.50
PVS3	0ºC	5	88.0±8.00	12.3±4.93
		15	84.3±6.02	22.0±3.00
	25ºC	5	85.0±10.58	23.0±5.29
		15	83.0±7.54	25.0±8.54

Note: *SD - standard deviation

As it is shown in Table 1, none of the three solutions used proved to be cytotoxic, so that in the case of control shoot tips a regeneration rate higher than 80% was obtained regardless of the temperature or duration of action of the solution.

In the case of the PVS1 solution, the highest regeneration rate of 34% was found for a treatment duration of 15 minutes at 25ºC. The cryoprotective effect of PVS2 was better than that of the PVS1 or PVS3 cryoprotective mixture. The use of the PVS2 solution resulted in higher regeneration rates in the case of cryopreserved shoot tips, compared to the other mixtures tested. Thus, in the case of a PVS2 treatment for 15 minutes, at a temperature of 0ºC, 42% of the shoot tips were regenerated, and in the case of a treatment for 15 minutes at a temperature of 25ºC, 49% of the shoot tips were regenerated. Vitrification using the PVS3 solution led to a

regeneration rate of 35% when treatment was carried out for 15 minutes, at a temperature of 25ºC.

The results obtained led us to the conclusion that the treatment carried out at the temperature of 25º was more efficient than that performed at 0ºC in all tested solutions. Also, a treatment duration of 15 minutes proved to be more efficient in obtaining cryoprotection. The finding that in the case of potato shoot tips the best results in terms of regeneration were obtained by using the cryoprotective PVS2 mixture made us pursue our studies in this context.

We also extended the duration of treatment with PVS2 to 30 minutes. Thus, the 20% and 60% concentrations were additionally tested for 30 minutes (Tab. 2).

The maintenance of the regenerative capacity of cryopreserved shoot tips is influenced by various parameters, such as concentration, duration of treatment with the cryoprotective mixture and temperature at which the treatment is performed.

Table 2: Influence of the PVS2 solution on the maintenance of the regenerative capacity of shoot tips (cv. Ostara) following cryopreservation (treatment duration - 30 minutes)

PVS2 (%)	**Temperature** (ºC)	Shoot regeneration (controls) (%)	Shoot regeneration (after cryopreservation) (%)
20%	0ºC	91.3±4.16*	15.6±5.03
	25ºC	89.6±5.03	26.6±4.93
60%	0ºC	88.6±7.02	29.0±8.18
	25ºC	90.3±6.65	34.0±5.56

Note: *SD - standard deviation

The fact that the best results regarding shoot regeneration following cryopreservation were obtained using high cryoprotective mixture concentrations was emphasized by Yamada et al. [16] and by Steponkus et al. [13]. The mentioned authors consider that as much as 90% of the water responding to osmotic treatment can be eliminated by this treatment. According to some authors [15, 11], short duration exposures of cells to cryoprotective treatment at 25ºC are more efficient than treatment at 0ºC.

Influence of the warming temperature on the maintenance of the regrowth capacity of cryopreserved shoot tips

Another step the role of which should not be neglected in ensuring the viability of shoot tips after cryopreservation is the warming of the cryopreserved shoot tips, more precisely the temperature and the nature of the warming medium. Shoot tips were submitted to the pre-freezing treatments described above. The influence of the warming medium on the regeneration of shoots from cryopreserved shoot tips is shown in Table 3. A regeneration rate of 43% was obtained when shoot tips were warmed in a liquid medium. It should be noted that warming in air resulted in a low regeneration rate (7%).

Table 3: Influence of warming temperature on the regrowth of plants from cryopreserved shoot tips (cv. Ostara)

Experimental series	Warming temperature (ºC)	Regenerated plants (%)
1.	25ºC (air)	7.6±3.51*
2.	25ºC (liquid medium)	43.6±7.63

Note: *SD – standard deviation

An important aspect of warming is represented by the dilution of the cryoprotective mixture and its subsequent removal in order to eliminate the possible negative effects caused by the high concentration of cryoprotective agents. The „washing" media used were solutions containing an osmotically active agent, such as sucrose [7, 9] or sorbitol [1]. The problem of the „washing" of the plant material after vitrification is quite controversial.

Conclusions

The results obtained led us to the conclusion that the treatment carried out at the temperature of 25ºC was more efficient than that performed at 0ºC in all tested solutions. Also, a treatment duration of 15 minutes proved to be more efficient in obtaining cryoprotection. The maintenance of the regenerative capacity of cryopreserved shoot tips is influenced by various parameters, such as concentration, duration of treatment with the cryoprotective mixture and temperature at which the treatment is performed.

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CRIOCONSERVAREA APEXURILOR DE CARTOF PRIN VITRIFICARE

(Rezumat)

Materialul vegetal utilizat pentru experimentele de crioconservare prin vitrificare a constat din apexuri caulinare de cartof (*Solanum tuberosum* L.). Acestea au fost supuse tratamentelor de vitrificare cu soluție PVS2 şi au fost congelate prin imersie directă în azot lichid (-196°C). Apexurile congelate au regenerat plantule la patru săptămâni de la decongelare şi transferul lor pe medii optime de regenerare. Regenerarea de plantule din apexurile crioconservate a fost diferită în funcție de temperatura la care s-a realizat tratamentul în vederea vitrificării conținutului celular în momentul contactului cu azotul lichid. În cazul unui tratament cu PVS2 efectuat la temperatura de 0ºC timp de 15 minute procentul de regenerare de plantule a fost de 42% iar în cazul în care tratamentul s-a realizat la temperatura de 25ºC tot timp de 15 minute regenerarea de plantule a fost de 49%. Ca mediu pentru regenerarea apexurilor culinare s-a utilizat mediul mineral Murashige-Skoog suplimentat cu giberelină 0,2 mg/l, zeatină 1 mg/l, acid indolilacetic 0,5 mg/l şi glicină 1 mg/l. Metoda de vitrificare utilizată poate fi aplicată cu succes pentru crioconservarea apexurilor caulinare de cartof.