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CRYOPRESERVATION OF CARNATION SHOOT APICES BY VITRIFICATION

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Abstract: This paper presents a protocol concerning cryopreservation of carnation shoot apices (2-4 mm) in liquid nitrogen for 48 hours. Vitrification of apices was performed by treating them with the PVS2 (glycerol 30% + EG 15% + DMSO 7%) vitrification solution. Different parameters were tested, such as: PVS2 concentration, i.e. 20%, 60% and 100% concentrations; the duration of action of the cryoprotective mixture: 5, 10, 15, 20, 25 and 30 minutes; as well as different incubation temperatures, 0±1°C and 25±1°C, respectively. The best results in the survival of carnation shoot apices were found when the 100% PVS2 solution was used, with an action duration of 10 minutes and treatment performed at the temperature of 0±1°C. Results were evaluated a month after thawing and transfer of apices to the MS regeneration medium [6] supplemented with α-naphthylacetic acid 0.2 mg/l and benzyladenine 1 mg/l.

Introduction

Vitrification in the context of cryopreservation consists in the process of transition of a water solution to a solid, vitreous, amorphous mass, phenomenon that occurs at/below the freezing temperature of the water solution [5,17]. Vitrification, as a method of cryopreservation, presents two important characteristics: the existence of extremely high cooling rates and the use of high concentrations of cryoprotective agents. The tolerance limit for cell dehydration ranges between 55-75% [13]. When this limit is exceeded, irreversible changes can appear in cells, because of the loss of integrity of cell membranes and penetration of ice crystals inside the cell.

Cryopreservation of carnation shoot apices was first performed by Seibert (1976). The author treated apices with 5% DMSO, after which these were immersed in liquid nitrogen, but the plantlet regeneration rate was only 5%. Later, the method was improved by storage of donor plants at the temperature of 4°C, use of high saccharose concentrations (0.75 M) [1] or gradual decrease in temperature [15].

In the present paper we studied the cryopreservation of carnation shoot apices (*Dianthus caryophyllus* L., cv. Pink Candy) by the vitrification method.

Material and methods

Shoot apex culture

The plant material used in experiments of cryopreservation by vitrification was represented by shoot apices (2-4 mm) taken from carnation in vitro plantlets.

The excision of shoot apices was performed under aseptic conditions, using the binocular microscope (10x, 20x), and sterile hypodermic needles (0.7x38 mm). After excision, the carnation shoot apices were transferred to sterile Petri dishes (5 cm in diameter), on aseptic filter paper, humidified with MS nutritive liquid medium [6], supplemented with α-naphthylacetic acid (NAA) 0.2 mg/l and benzyladenine (BA) 1 mg/l (C). Apices were incubated in the same medium C for 24 hours, in order to recover from the stress caused by their detachment from the explant producing plantlets.

The vitrification process

For freezing, apices were treated with 0.5 M saccharose solution for 24 hours. Later, they were incubated in high concentration cryoprotective solutions for osmotic cell dehydration. The following vitrification solutions were used: PVS1 [17], PVS2 [11] and PVS3 [7]. Apices were incubated in cryoprotective solutions for 5 to 30 minutes, after which they were frozen in the same solutions, by direct immersion in liquid nitrogen (LN_2). Due to the extremely high concentrations of cryoprotective agents, ice crystals do not form during freezing. Thawing was performed in water bath at the temperature of 40°C. After thawing, apices were transferred to the C semisolid medium (3% agar) in order to be regenerated. Culture conditions consisted in a photoperiodic regime of 16 hours light/24 hours, intensity 40 $\mu E.m^{-2}.s^{-1}$, temperature 25°C.

Three repeats of 25 apices each were carried out for each experiment. Results were evaluated one month after thawing and transfer of apices to the regeneration medium.

Results and discussion

Effect of loading on the survival of apices

Due to the fact that the vitrification technique eliminated the gradual decrease of temperature which ensured the protection of cells during dehydration, a compensatory method of dehydration was necessary. In this respect, plant material was exposed to the action of high concentrations of cryoprotective agents. In order to avoid osmotic shock, which might have caused cell death, this treatment was performed in two stages. In a first stage, shoot apices were exposed to the action of cryoprotective substances in lower concentrations (maximum 1M). In order to find the optimum cryoprotective agents, the effects of the following cryoprotective substances were tested: dimethyl sulfoxide (DMSO), glycerol, ethylene glycol (EG) or polyethylene glycol (PEG). After these treatments, apices were incubated in 100% PVS2 solution for 15 minutes, then they were frozen by direct immersion in liquid nitrogen. The various types of loading mixtures, destined to ensure

cryoprotection and viability of apices during their cryopreservation by the vitrification method, are shown in Table 1.

Table 1: Effect of loading solutions on the survival of vitrified carnation apices cooled to -196ºC following dehydration with PVS2.

Loading solutions	Survival (% ± s.d.)*	
	Treatments at 0ºC	**Treatments at 25ºC**
DMSO 10%	0	0
EG 10%	0	0
PEG 10%	0	0
Glycerol 10%	33 ± 3	24.6 ± 4.5
Saccharose 0.4 M + DMSO 10%	33.3 ± 4	21.6 ± 4
Saccharose 0.4 M + DMSO 5%	31 ± 5	16.6 ± 5
Saccharose 0.4 M + glycerol 0.5 M	64.6 ± 4.1	48.6 ± 5.6
Saccharose 0.4 M + glycerol 1 M	67.6 ± 2	50.3 ± 3.5
Saccharose 0.4 M + EG 0.5 M	68.6 ± 3	53 ± 3.5
Saccharose 0.4 M + EG 1 M	63 ± 3.6	56.3 ± 2.5
Saccharose 0.4 M + EG 1 M + DMSO 5%	35.3 ± 4	51.3 ± 4.1
Saccharose 0.4 M + EG 0.5 M + DMSO 10 %	48 ± 4.5	32 ± 4.5
PEG 10% + EG 1 M + DMSO 5%	22.6 ± 3	9.6 ± 3.5
PEG 20% + EG 0.5 M + DMSO 10%	8.3 ± 3.5	6 ± 3.5

Note: * s.d. – standard deviation

Apices were loaded with each solution for 10 minutes at 0ºC and at 25ºC and then dehydrated with PVS2 at 25ºC for 15 minutes prior to immersion in LN_2. Treatments with loading substances were applied for the concentration of protoplasm, which is indispensable for successful cryopreservation by vitrification.

Table 1 shows that treatment with cryoprotective substances at the temperature of 25°C resulted in lower regeneration rates than treatment at 0°C. Cryoprotective substances used individually (DMSO, EG or PEG) did not ensure the maintenance of viability of shoot apices, following cryopreservation in liquid nitrogen regardless of temperature. The highest regeneration rates, 68%, were obtained by using the mixture saccharose 0.4 M + EG 0.5 M, and 67% with the mixture saccharose 0.4 M + glycerol 1 M, treatment carried out at the temperature of 0°C. Low regeneration rates were also found in the case of the mixture containing PEG 20%. Thus, in the case of the mixture consisting of PEG 20% + EG 1 M + DMSO 10% M, survival rates were 8% when treatment was performed at 0°C and 6% when treatment was performed at 25°C. We mention that DMSO in combination with saccharose did not ensure adequate cryoprotection regardless of temperature.

Authors believed that the time duration necessary for the equilibration of concentrations between the intracellular and extracellular environment is 5-25 minutes, depending on the temperature at which this operation occurs [3]. Opinions

are divided whether a single cryoprotective agent or a mixture of several cryoprotective substances should be used [9,3,4,2,10]. However, the importance of EG for the ensurance of viability after cryopreservation by vitrification is emphasized [14,17].

Following treatments with loading solutions, as described before, the cell content was concentrate to tolerate exposure to solutions with even higher concentrations for extreme cell dehydration.

Effect of vitrification on the survival of apices

After treatments with loading solution (0.4 M + EG 0.5 M) carnation apices were treated with PVS1, PVS2 and PVS3 solutions at the indicated temperature and for the indicated time.

The results obtained following treatment with the cryoprotective mixtures (PVS1, PVS2 and PVS3) are shown in Table 2.

Table 2: Effect of treatments used to expose carnation apices to PVS1, PVS2 and PVS3 solutions on the survival of apices cooled to –196ºC by vitrification.

Vitrification solutions	Treatments temperature **(ºC)**	**Time (minutes)**	Survival **(% ± s.d.)***
Experiment 1			
PVS1 solution (Uragami et al. 1989) - glycerol 22% (w/v) - EG 15% (w/v) - propylene glycol 15% (w/v) - DMSO 7% (w/v)	0ºC	5	52.3±3.2
		15	46.3±4.0
	25ºC	5	44.0±4.0
		15	40.6±3.0
Experiment 2			
PVS2 solution (Sakai and Kobayashi 1990) - glycerol 30% (w/v) - EG 15% (w/v) - DMSO 7% (w/v)	0ºC	5	57.0±5.0
		15	55.0±3.6
	25ºC	5	51.3±4.1
		15	47.6±4.0
Experiment 3			
PVS3 solution (Nishizawa et al. 1993) - glycerol 30% (w/v) - saccharose 50% (w/v)	0ºC	5	54.3±4.0
		15	54.0±3.6
	25ºC	5	56.6±3.5
		15	50.6±4.0

Note: *s.d. – standard deviation

Table 2 indicates that the use of PVS1 solution ensured a survival rate of 52% for a treatment carried out at the temperature of 0°C for 5 minutes, compared to 44% with the same duration of treatment, at the temperature of 25°C. The use of the PVS2 solution resulted in higher survival rates of cryopreserved apices compared to the other tested mixtures. Thus, in the case of a PVS2 treatment for 5

minutes, at 0°C, 57% of the control apices survived 30 days after treatment and their inoculation on the optimal medium for regeneration (experiment 2).

When treatment was carried out at the temperature of 25ºC, for 5 minutes, 51% of the apices survived. In this case, however, the extension of the treatment duration to 15 minutes led to a decrease in the regeneration rate to 47%. When the PVS3 mixture was used, the survival rate was not affected by this cryoprotective mixture treatment, so that regeneration rates higher than 50% were found both at the temperatures of 0ºC and 25ºC and for the durations of 5 and 15 minutes.

The finding that in the case of carnation apices the best results in regeneration were obtained by using the PVS2 cryoprotective mixture made us include in our study another parameter, that of monitoring the cryoprotective efficiency of other concentrations of the PVS2 solution, 20%, 60% and 100% respectively, for different time periods ranging between 5 and 30 minutes.

The influence of PVS2 on the maintenance of the regenerative capacity of carnation shoot apices following cryopreservation is presented in Fig. 1-6. By the application of a 20% PVS2 concentration (Fig. 1, 2), non-frozen shoot apices present an insignificant decrease in the regeneration rate when the action duration of the PVS2 solution is prolonged, at both tested temperatures, 0ºC and 25ºC, respectively. Cryopreserved shoot apices show a similar evolution at the two tested temperatures. The analysis of Fig. 1 shows a gradual increase of the regeneration process with the extension of the treatment duration to 25 minutes, when a 29% rate is found at the temperature of 0ºC. The same treatment carried out at the temperature of 25ºC determines a similar evolution of the regenerative capacity of the cryopreserved apices, a 28% survival rate being found in the case of a 20 minute treatment (Fig. 2).

The application of a treatment with 60% PVS2 mixture in the case of (non-frozen) control apices caused an evolution characterized by a slight decrease in the regeneration process with the prolongation of the action duration of the cryoprotective mixture (Fig. 3, 4). In the case of apices cryopreserved with 60% PVS2, a gradual increase in the regeneration process was found when the treatment duration was extended up to 20 minutes, at which point a 62% survival rate was found for treatments carried out at the temperature of 0ºC (Fig. 3). Treatment for the vitrification of cell content performed at the temperature of 25ºC resulted in an evolution of regeneration similar to that found at 0ºC, but only for a duration of up to 15 minutes, when a 57% survival rate was obtained, after which the regeneration rate decreased to 21%, for a 30 minute duration action of PVS2 (Fig. 4).

By using PVS2 in a 60% (Fig. 3, 4) concentration, significant differences were found in plantlet regeneration between the two tested temperatures of 0ºC and 25ºC, for a 20 minute duration of treatment ($P < 0.5$).

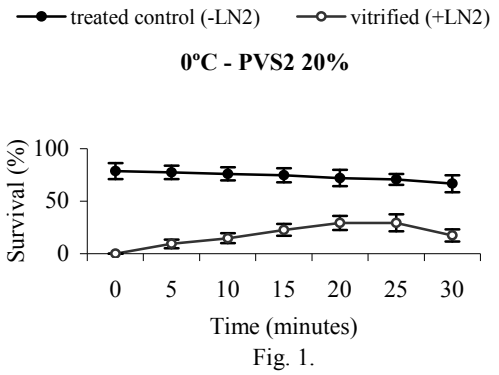

Fig. 1.

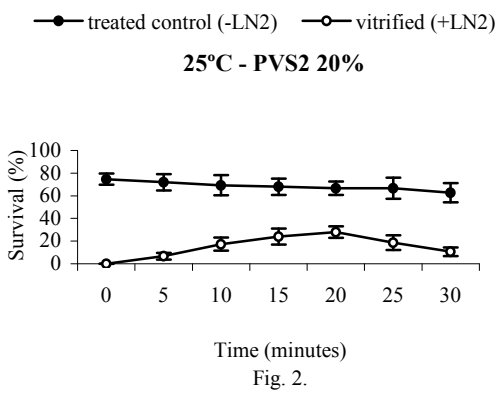

Fig. 2.

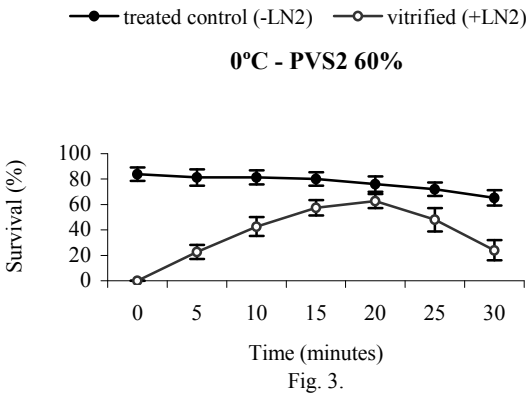

Fig. 3.

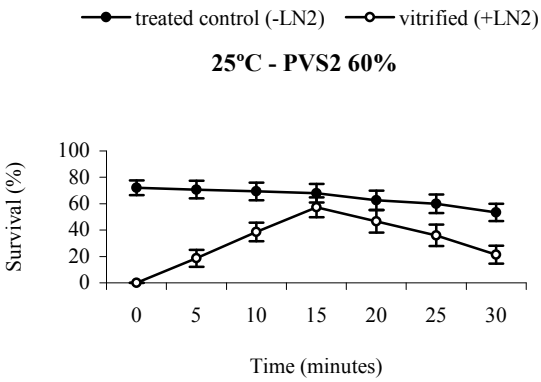

Fig. 4.

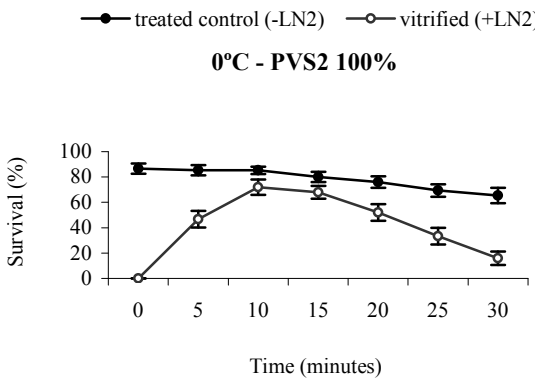

Fig. 5.

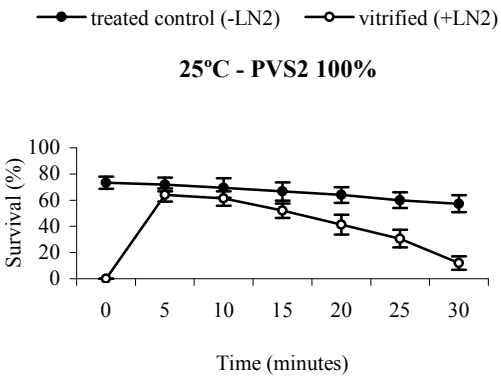

Fig. 6.

Fig. 1-6: Effect of exposure time to PVS2 at 0ºC or 25ºC on the survival of carnation apices cooled to -196ºC by vitrification. Apices were loaded with a mixture of 0,4 M sucrose and 0,5 M etylenglycol in MS medium at 25ºC for 15 minutes and than dehydrated with PVS2 for various lengths of time at 0ºC or 25ºC prior to a plunge into LN. Vertical bars represents standard deviation.

The use of a 100% cryoprotective PVS2 mixture resulted in the case of non-frozen vitrified control apices in a slight decrease in the regeneration rate when the duration action of the vitrification solution was extended from 5 minutes to 30 minutes, both at the temperature of 0ºC and 25ºC (Fig. 5, 6). The regeneration rate of cryopreserved apices reached a maximum value, 72%, in the case of treatment applied for 10 minutes at the temperature of 0ºC, and 64% for a treatment with a 5

minute duration at the temperature of 25ºC. The use of the PVS2 cryoprotective mixture in a 100% concentration led to significant differences in the regeneration of plantlets from the cryopreserved apices between the two tested temperatures, 0ºC and 25ºC, for a treatment duration of 10 minutes ($P < 0.01$) and 15 minutes ($P < 0.02$).

The fact that the best results in regeneration following cryopreservation by vitrification were obtained with high concentrations of cryoprotective mixtures was emphasized [18,13,8,16]. Other authors considered that at 0ºC, cryoprotective substances cannot penetrate the cell, on the one hand because of the difference in permeability between water and cryoprotective solutions and, on the other hand, because of the important difference between the activation energy consumed for the penetration of water and the energy consumed for the penetration of cryoprotective solutions in the cells [13].

Conclusions

The maintenance of the regenerative capacity of shoot apices cryopreserved by vitrification was influenced by two parameters, i.e. the concentration and duration of action of the cryoprotective mixture.

Of the three PVS2 concentrations tested, 20%, 60% and 100%, the best results in the survival of carnation shoot apices were found using the 100% PVS2 concentration. Regarding the duration of action and the temperature at which treatment with 100% PVS2 was performed, the best results were obtained for a 10 minute duration, at 0°C.

Cryopreservation by vitrification of carnation shoot apices is possible in the variety studied, following administration of correct treatments prior to freezing.

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CRIOCONSERVAREA APEXURILOR CAULINARE DE GAROAFE PRIN VITRIFICARE

(Rezumat)

Lucrarea prezintă un protocol privind crioconservarea apexurilor caulinare de garoafe (2-4 mm) în azot lichid timp de 48 de ore. Vitrificarea apexurilor s-a realizat prin tratarea acestora cu soluția de vitrificare formată din glicerol (g/v) 30% + EG 15% (g/v) + DMSO 7% (g/v) (PVS2). Au fost testați diferiți parametri, cum sunt: concentrația de PVS2, caz în care au fost testate următoarele concentrații: 20 %, 60 % şi 100 %; durata de acțiune a amestecului crioprotector, care a fost de 5, 10, 15, 20, 25 şi 30 minute, precum şi diferite temperaturi de incubare respectiv de 0±1ºC şi de 25±1ºC. Cele mai bune rezultate privind supraviețuirea apexurilor caulinare de garoafe s-au înregistrat în urma utilizării PVS2 în concentrație de 100% la un timp de acțiune de 10 minute şi un tratament efectuat la temperatura de 0±1ºC. Evaluarea rezultatelor s-a realizat la o lună după decongelare şi transferul apexurilor pe mediul de regenerare MS (Murashige and Skoog, 1962) suplimentat cu acid ∝-naftilacetic 0,2 mg/l şi benziladenină 1 mg/l.