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Post-Thaw Survival of Meristems from *in vitro* Sweet Potato (Ipomoea Batatas (L.) Lam.) Plants †

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Abstract: Cryotherapy of shoot tips can effectively eliminate the sweet potatoes pathogens, such as viruses and phytoplasm and is impossible without the development of effective cryopreservation techniques. At the same time, cryopreservation allows a long-term germplasm storage. In this study, we developed and compared different methods for sweet potatoes meristems treatment for the optimized preservation. The meristems of Admiral variety up to 1–2 mm were isolated from *in vitro* growth plants. In one group the specimens were dehydrated 120 min with sterile air flow and immersed into liquid nitrogen at a needle tip. The meristems of other groups were dehydrated with plant vitrification solutions (modified PVS 1, PVS 2, 88% PVS 3, and PVS N). The samples were immersed into liquid nitrogen either in 1.8 mL cryovials or 50 µl hermetically sealed aluminum pans for differential scanning calorimetry. It was shown that the survival rates of meristems were 55% after 88% PVS 3 treatment and 83 and 85% after PVS 2 and PVS N exposure. The highest percentage of preserved specimens was found after dehydration with air-flow and modified PVS 1 (89–95%). After cryopreservation in 1.8 mL cryovials the highest post-thaw preservation was noted after pretreatment with modified PVS 1 (60–75%), the lowest one was observed with 88% PVS 3 (35–40%). Meristems treated with PVS 2 and PVS N provided the 45–55% survival rates. After cryopreservation in aluminum pans the highest post-thaw preservation was detected for dehydration with modified PVS 1 (81–84%), the lowest one was found with 88% PVS 3 (40–51%). Meristems treated with PVS 2 and PVS N revealed of 68–78% post-thaw survival respectively. The meristems cryopreservation method based on dehydrated with the sterile air flow is of a special interest, since no cryoprotectant use is needed.

Keywords: cryopreservation; sweet potato; vitrification; plant vitrification solution; air dehydration

1. Introduction

The introduction of vegetable crops with a high content of biologically active compounds into agriculture remains a relevant task. Sweet potato (*Ipomoea batatas*) is the seventh most important food crop grown in many countries. Its root tubers are rich in sugars, which determine its taste, as well as in proteins, B vitamins, ascorbic acid, carotenoids, macro- and microelements [5]. Sweet potato is vegetatively propagated, and a consistent supply of virus-free planting material is critical for

sustainable production. Cryotherapy is a promising procedure for eliminating pathogens from infected plant tissue. Cryopreservation is widely used for the long-term preservation of plant genetic resources. The standard procedure usually involves cutting out the shoot tips, immersing them into liquid nitrogen and, after thawing and post-cultivation, regenerating into plants. Recently, cryotherapy has been demonstrated to eradicate seven unrelated groups of viruses and two types of bacterialike pathogens from several species of economic importance, i,e. *Prunus*, *Musa spp.*, *Vitis vinífera, Fragaria ananassa*, *Solanum tuberosum*, *Rubus idaeus*, *Ipomea batatas*, *Dioscorea opposite* and *Allium sativum* [1,19]. For cryotherapy of plants, cooling conditions must allow survival of the cells only in apical dome and in the youngest (1st–2nd) leafs primordia. The basal part of the apical dome and more advanced leafs primordia are to be killed by liquid nitrogen (LN) as far as these cells are generally infected by plant pathogens, in particular viruses [19]. Shoot tips used for cryotherapy can be relatively large thus; they can show relatively high regenerative capacity [1]. Based on previously published data, cryotherapy can be used as a proper and easily implemented approach for eradicating various pathogens from plant tissue, and can replace traditional methodologies [18]. Cryotherapy has a number of advantages compared to traditional approaches, i.e., simplicity and time efficiency, ability to treat simultaneously a large number of samples ; low cost and high frequency of plants free from viruses after recovery [1].

The greatest challenge for the wider application of cryotherapy is that different genotypes of the same species may differently respond to cryotherapy [2,17,19]. Several protocols with different cryotherapy methods have been reported and the results differ between laboratories, with varying levels of survival and regeneration [1]. Since the cryotherapy is impossible without the effective techniques of cryopreservation, in this work, we compared efficacy of different cryopreservation technique for the sweet potatoes meristems (Admiral cultivar) preservation.

2. Experiments

Sterile explants were obtained from the shoots of sprouted Admiral cultivar sweet potato tubers. Parts of the stems were sterilized in 30% sodium hypochlorite solution for 25 min and washed 5 times with sterile distilled water. Then they were transferred to vials with agar nutrient medium Murashige and Skoog (MS) [7], supplemented with 3% sucrose, 6% agar vitamins and 0.1 mg/l benzylaminopurine, 0.5 mg/l naphthylacetic acid and 2.0 mg/l gibberellic acid. To obtain a sufficient number of samples, the in vitro shoots were divided into parts, each containing a stem with a leaf and a lateral bud. The cuttings were planted in vials with solid nutrient medium MS, supplemented with 3% sucrose, 6% agar and 0.01 mg/l indoleacetic acid. The in vitro material was cultured at 20 ± 2 °C, with 16 h of light and 8 h of darkness and 2 kilolux light intensity. The explants were propagated by micro-grafting every 45 days.

For cryopreservation the apical and axillar meristems up to 1–2 mm were isolated from three-week in vitro cultured plants. The isolated samples were transferred into a liquid MS medium, supplemented with 12% sucrose and exposed at dark for 24 h. Prior freezing the meristems were divided into the experimental groups. The first group contained meristems dehydrated for 120 min with sterile airflow (AD), frozen by plunging into LN on a needle tip, and re-warmed in MS medium, enriched with 12%sucrose. Other groups consisted of meristems incubated for 60 min at 22 °C with the following cryoprotectant solutions: (i) PVS 2 (30% glycerol + 15% ethylene glycol + 15% dimethyl sulfoxide and 0.4 M sucrose [11]), (ii) 88% PVS 3 (44% glycerol + 44% sucrose [8]), (iii) modified PVS 1 (22% glycerol + 13% 1,2-propylene glycol + 13% ethylene glycol + 6% dimethyl sulfoxide and 0.4 M sucrose [14]) and (iv) PVS N (1 M sucrose + 15% glycerol + 14% ethylene glycol [4]). The meristems of all groups after PVSs treatment were cooling 1.8 mL cryovials («Corning», USA) or 50 μl hermetically sealed aluminum pans for differential scanning calorimetry (DSC) and were directly immersed into LN for 1 h. The specimens were re-warmed in water bath at 40 °C. The cryoprotectants were removed by two subsequent transfers of the meristems in fresh medium MS, enriched with 0.3 M sucrose. To examine the influence of the pretreatment steps on the explants the part of meristems were treated excluding low-temperature exposure. The meristems that were not dehydrated or

cooling were selected as a control group. The post-thaw explants of all the groups were transferred into the agar MS medium, supplemented with 3% sucrose, 3 mg/l gibberellic acid and 0.01 mg/l indoleacetic acid. The survival was determined by the number of meristems, which had a green color for 30 days. In all experiments, 15–25 meristems were used per experimental condition and the experiments were replicated 3–4 times. The results were statistically analyzed using Software Past 3. The results are presented as arithmetic mean and standard deviation. For establishing statistical significance we used non-parametric Mann-Whitney criterion. The difference was considered statistically significant at $p < 0.05$.

The phase and glass transitions of PVSs in the temperature range from –196 °C to complete media melting were investigated using low-temperature DSC [12]. Glass transition temperature (Tg) was determined as a midpoint between the onset and endset of the inflectional tangent. Crystallization (Tc) and melting (Tm) temperatures were determined as an extrapolated onset-temperature (the designed point of intersection of the extrapolated baseline and the inflectional tangent at the beginning of the melting or crystallization peak). In this study, the samples were frozen by immersion into LN, with an average cooling rate of 200 degrees/min. The thermograms were recorded during warming with the rate of 0.5 degrees/min. The weight of all the investigated samples was 1 g.

3.1. Subsection

For successful cryopreservation, it is essential to avoid the lethal intracellular freezing that occurs during rapid cooling in LN. Thus, in any cryogenic procedure, the specimen must be sufficiently dehydrated to avoid freezing and to allow vitrification upon rapid cooling in LN. Currently, the most popular cryogenic procedures are «vitrification» and «encapsulation/dehydration» [6]. The difference between the two methods is the method of dehydration. In «vitrification», cells are dehydrated with a highly concentrated cryoprotective solution, in «encapsulation/dehydration» – air-drying; then, dehydrated samples can be vitrified by rapid cooling (immersion into LN).

For successful dehydration, additional pretreatment of the meristems is often carried out by preliminary cultivation on a nutrient medium. Sucrose preculture was determined to be necessary for the adaptation of sweet potato shoot tips to cryoprotection with PVS and highly significantly affected survival of sweet potato meristems after cryopreservation [9]. In our studies, the meristems were preliminarily cultured in a nutrient medium MS with the addition of 12% sucrose and kept under dark conditions. After such an exposure, all the meristems formed plantlets during further cultivation with a MS medium.

An important indicator of successful cryopreservation is a high level of meristems preservation after pre-treatment before immersion into LN. It was shown that the survival rate meristems did not exceed 55% after treatment with 88% PVS 3. The preservation rates after PVS 2 and PVS N exposure were 83 and 85% respectively. The highest number of preserved specimens was noted after dehydration with airflow (89–95) and modified PVS 1 (88–94%). Since a decrease in the viability of the meristems was also observed in the control meristems (88%), most likely it was connected with their damage during isolation (Figure 1).

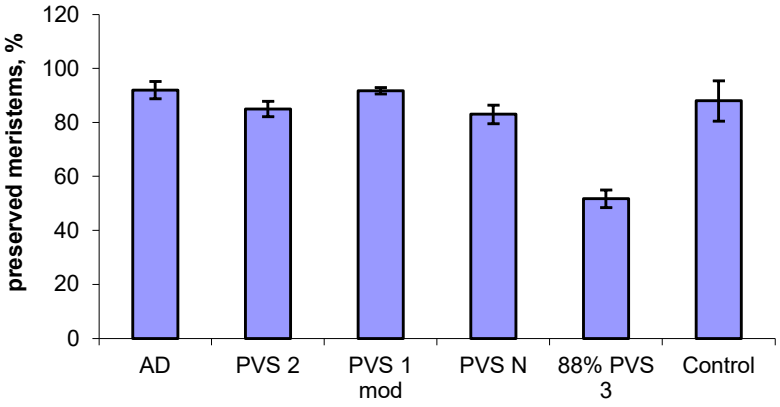

Figure 1. Preservation rate of sweet potato meristems after different pretreatments before immersion into LN: AD—dehydration with sterile airflow; PVS 2, mod PVS 1, PVS N, 88% PVS 3—dehydration with appropriate plant vitrification solution; Control—non-treated meristems; *—differences are significant if compared with the control group, $p < 0.05$.

The decrease in the number of viable meristems after exposure in 88% PVS 3 may be associated with the toxic effects of high concentrations of cryoprotectants or with osmotic reactions that lead to damage of specimens.

The meristems dehydrated with sterile airflow provided a preservation level about 85% after warming, which was not significantly different from non-cooling ones.

After cryopreservation in cryovials, the meristems survival rates of 35 – 75% were observed (Figure 2). The highest post-thaw preservation was found in pretreatment with modified PVS 1 (60–75%). The lowest survival rate was observed in case with 88% PVS 3 (35–40%). Meristems treated with PVS 2 and PVS N demonstrated a survival index of 45–55%.

After cryopreservation in hermetically sealed aluminum pans for DSC the meristems preservation rates of 40–82% were observed (Figure 2). The highest post-thaw preservation was found for pretreatment with modified PVS 1 (81–84%). The lowest survival rates were observed in the case with 88% PVS 3 (40–51%). The meristems treated with PVS 2 and PVS N showed survival indices of 68–75 and 70–78% respectively.

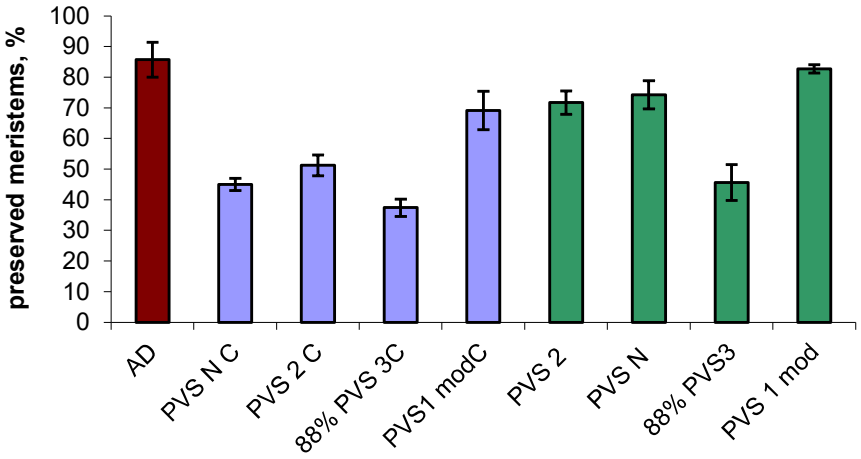

Figure 2. Survival rate of cryopreservation sweet potato meristems: AD – immersion into LN at a needle tip; PVS 2 C, PVS 1 mod C, PVS N C, 88% PVS 3C for 1.8 mL cryovials; PVS 2, PVS 1 mod, PVS

N, 88% PVS 3 for 50 μl hermetically sealed aluminum pans for DSC; *—differences are significant if compared with the control group, $p < 0.05$.

4. Discussion

According to our results, the use of aluminum pans for DSC significantly determined higher survival rate of cryopreserved sweet potato meristems, compared to those of the vitrification using cryovials. We believe it may depend on different cooling and warming rates in these containers. The cooling rate at the step from 22 down to –70 °C made 49 ± 12 deg/s for 1.8 mL cryovials and 125 ± 14 deg/s for the 50 μL aluminum pans for DSC. The rate of warming from –196 to 22 °C was 42 ± 15 for the cryovials and 348 ± 26 deg/s for the metal container.

The high meristems preservation rate observed by us most likely associated with the stability of the vitrified state of modified PVS 1. This was shown by differential scanning calorimetry, recorded during warming with the rate of 0.5 deg/min (Figure 3).

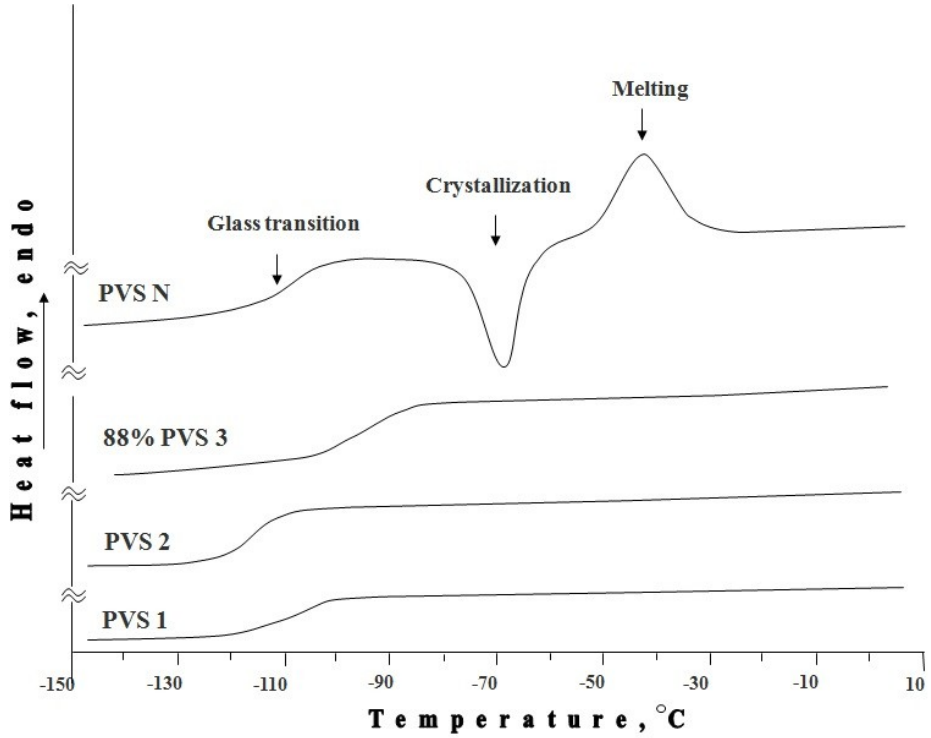

Figure 3. DSC termogram of different plant vitrification solutions.

DSC thermograms of modified PVS 1, PVS 2 and 88% PVS 3 revealed only one heat capacity jump at temperature Tg (–109; –115.3; –93.9 respectively), associated with a reverse glass transition process (transition from a solid amorphous to of supercooled liquid state). No exo- or endothermic peaks were recorded, indicating no crystallization, both at cooling and warming stage. This fact testifies that at the cooling stage the modified PVS 1, PVS 2, and 88% PVS 3 are completely transformed into a glassy state with highly stable amorphous phase, which does not crystallize even under slow warming above the glass transition temperature. In the thermogram of PVS N, in addition to the glass transition, an exothermic crystallization from an amorphous state (devitrification) peak and an endothermic peak of melting were recorded (Figure 3). It should be noted that the area under crystallization and melting curves does not differ significantly.

This indicates that the crystallization occurs only at the warming stage. In spite of the fact that in the PVS N there were recorded the devitrification and endothermic peaks of melting at warming stage, we obtained the survival rate of meristems at the level of 42–79%. This is due to the fact that the heating rates we applied in our studies were quite high. Since PVS 3 had a negative effect on

meristems at the treatment stage, its stable amorphous phase did not affect the preservation rate of the meristems after cryopreservation.

Cryopreservation of sweet potato meristems for long-term storage of germplasm and cryotherapy is carried out by vitrification [10]; encapsulation vitrification [3], encapsulation dehydration [20] and droplet vitrification [9,10,15].

The date obtained [10] showed that despite the high level of survival, the regeneration may be absent. Vollmer and co-workers reported about successful cryopreservation of apical meristems with a regeneration rate from 1.7 to 66% for 24 tested sweet potato cultivars [15]. The date obtained [16] for tested cultivars showed regeneration rates of cryopreserved meristems ranging from 9.5 to 83.9%.

In view of the above, we are going to develop cryopreservation protocols with high level of regeneration for sweet potato varieties grown in Ukraine, which will be used to obtain virus-free plants (cryotherapy). Since the highest survival rates of sweet potato meristems after dehydration with airflow and PVS 1 and 2, we will use these protocols in further work. It is also necessary to select post-cultivation conditions, such as phytohormone composition, ammonium concentration in the nutrient medium and lighting conditions.

5. Conclusions

The survival rates of the sweet potatoes' meristems after PVS 3, PVS 2 and PVS N treatment were 51.75, 85 and 83% correspondingly. The highest percentage of preserved specimens was detected after dehydration with airflow or modified PVS 1–92%.

The meristems dehydrated with sterile airflow provided 85% survival rate after warming. Meristems treated with 88% PVS 3, PVS 2 and PVS N demonstrated a survival index of 37.4 and 51,25 and 45% after cryopreservation in cryovials. The highest post-thaw survival was found after dehydration with modified PVS 1–69%. The results demonstrated that the post-thaw survival after cryopreservation in aluminum pans for DSC did not change compared with non-cooling but dehydrated with PVSs meristems (82.75% for modified PVS 1, 74.25–PVS N, 45.7–88% PVS 3 and 71.75–PVS 2).

The use of aluminum pans for DSC significantly determined higher survival rate of cryopreserved sweet potato meristems, compared to those of the vitrification using cryovials. It may depend on different cooling and warming rates in these containers.

At the cooling stage the modified PVS 1, PVS 2, and 88% PVS 3 are completely transformed into a glassy state with highly stable amorphous phase, which does not crystallize even under slow warming above the glass transition temperature. In the thermogram of PVS N, in addition to the glass transition, an exothermic crystallization from an amorphous state (devitrification) peak and an endothermic peak of melting were recorded. The area under crystallization and melting curves does not differ significantly.

This indicates that the crystallization occurs only at the warming stage. In spite of the fact that in the PVS N there were recorded the devitrification and endothermic peaks of melting at warming stage, we obtained the survival rate of meristems at the level of 45 and 74.25%. This is due to the fact that the heating rates we applied in our studies were quite high. Since PVS 3 had a negative effect on meristems at the treatment stage, its stable amorphous phase did not affect the preservation rate of the meristems after cryopreservation.

The meristems cryopreservation method based on dehydrated with the sterile air flow is of a special interest, since no cryoprotectant use is needed.

Author Contributions: S.N., B.O., I.T. and conceived and designed the experiments; S.N., B.N., M.A., K.G., and B.O. perfomed the experiments; I.T., B.O. and S.N. statistical data processing and analysis of results; B.N., M.A. I.T. contributed reagents, materials and analysis tools; S.N., B.O. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript

DSC	differential scanning calorimetry
LN	liquid nitrogen
MS	nutrient medium Murashige&Skoog
PVS	plant vitrification solution
Tg	glass transition temperature
Tc	crystallization temperature
Tm	melting temperature

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