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CRYOPRESERVATION OF SHOOT TIPS AND POLLEN OF POTATO

ABSTRACT

Cryopreservation is a frequently used method of long-term storage of potato meristems and pollen in liquid nitrogen (LN) in temperature of -196°C. This technique allows for theoretically unlimited storage of potato material. The most popular method of potato shoot tips preservation is cryopreservation by the solidification of liquids without crystallization (vitrification).The best method of pollen conservation is its direct immersion in LN. The successful regeneration after vitrification is genotype-dependent, which require optimization of protocol.

Key words: meristems, liquid nitrogen, long-term storage, pollen, vitrification

INTRODUCTION

Cryopreservation is considered as the best method of long-term storage of frozen biological material in a very low temperature, usually in liquid nitrogen (LN) (-196°C) (Kryszczuk, 2002; Mikuła and Rybczyński, 2006). Cryopreservation is especially useful to store gene resources of vegetative reproductive plants (Kaczmarczyk *et al.*, 2008), including potato. It is an alternative approach for field and *in vitro* collections of vegetatively propagated plant species (Kryszczuk *et al.*, 2006). The low temperature causes that all cell divisions and metabolic processes are stopped, allowing for theoretically unlimited storage of plant material (Gonzalez-Arnao, 2008). The advantage of this method is also the low cost and small area needed to store the material (Mikuła and Rybczyński, 2006). Disadvantage of cryopreservation technique is the varying regeneration of meristems, sometimes relative-

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ly low, depending on species and genotype (Dhital *et al.*, 2009). Most commonly stored in liquid nitrogen are plant seeds, somatic embryos, calluses, protoplasts, or cell suspensions. In the case of potato, the youngest shoot tips are the most frequently explored. Meristematic cells are resistant to low temperatures because they contain a small number of vacuoles and have a thick cytoplasm, with small water content (Belokurova, 2010). The material intended for freezing should be very young and in the same development phase, free from pathogens and usually comes from *in vitro* plants (Kryszczuk, 2002). Commonly used method of potato shoot tips preservation is cryopreservation by the process of vitrification, which mean solidification of liquids without crystallization. In the process of vitrification plant material is incubated in a cryoprotectants solution and then rapidly immersed in liquid nitrogen, which prevents the formation of tissue-destroying crystals (Mikuła and Rybczyński, 2006). The most commonly used cryoprotectants include: dimethyl sulfoxide (DMSO), glycerol, propylene glycol, polyethylene glycol and sugars (Kryszczuk, 2002). The final step of cryopreservation is to check the capacity of frozen explants for regeneration. Explants are usually defreeze at room temperature or in warm water (35-40°C). Defrosting should be quick to avoid renewed ice crystals formation. In IHAR-PIB, Młochów Research Center shoot tips of diploid potato are preserved in LN using vitrification method and pollen of potato by direct freeze in LN. The efficiency of meristem regeneration is varied and dependent on the genotype. The level of regeneration in IHAR-PIB Młochów is between 10 and 70% for respective genotypes. Thus, the procedure is modified for individual genotypes in order to increase the efficiency of regeneration (Smyda, 2011).

MATERIALS AND REAGANTS

1) Agar (Sigma – Aldrich, cat. No A1296)
2) Cryo-tubes 1.5 ml (Roth)
3) Dimethyl sulfoxide (DMSO) (Sigma – Aldrich, cat. No 276855)
4) Ethanol 96% ((Avantor Performance Materials Poland S.A, POCH, cat. No 396420113)
5) Ethylene glycol (Sigma – Aldrich, cat. No 324558)
6) Gibberellic acid (Sigma – Aldrich, cat. No G7645)
7) Glass tubes
8) Gloves, nitrile
9) Glycerol (Sigma – Aldrich, cat. No 49767)
10) Indole-3-acetic acid Sigma – Aldrich, cat. No I5148)
11) Liquid nitrogen
12) MS medium according to Murashige and Skoog, 1962
13) Permanent marker
14) Petri dishes (Roth)
15) Pipette tips (Eppendorf)
16) Plastic boxes for freezing in liquid nitrogen (Roth)

17) Scalpel blade (Swann-Morton, carbon Steel Surgical blades)
18) Scalpel handles (Weldon Instruments)
19) Zeatin riboside (Sigma – Aldrich, cat. No Z3541)

REAGENTS/SOLUTIONS

1) MS medium with 10.3% sucrose and 0.8% agar.
2) LM solution - 13.7% sucrose and 18.4% glycerol in MS medium, autoclaved.
3) PVS2 solution - 7% sucrose, 30% glycerol, 15% ethylene glycol, 15% DMSO in MS medium, autoclaved. (PVS2 solution can be stored at 4°C for about one week)
4) Liquid MS solution with 41.8% sucrose.
5) MSTo medium – MS medium with 0.5 mg × l^{-1} indole-3-acetic acid, 0.5 mg × l^{-1} zeatin riboside, 0.2 mg × l^{-1} gibberellic acid, 3% sucrose. Hormones are added into autoclaved MS medium.

EQUIPMENT

1) Autoclave (Prestige Medical, model Extended Plus 2100)
2) Climatic chamber for *in vitro* plants with controlled light and temperature
3) Dewar with liquid nitrogen
4) Laminar flow hood II class
5) LN tanks for long-term storage
6) pH meter
7) Pipette (Eppendorf)
8) Stereoscopic microscope (Motic)
9) Water purification system (Purelab, Elga)

PROCEDURE

Cryopreservation of potato shoot tips

1) *In vitro* plants are cut into segments containing one node each and placed on solid MS medium on Petri dishes and cultivated at 21°C/8°C (day/night), under 8 h illumination for one week. The target number of shoot tips intended for freezing is 50 – 60. In one cycle of cryopreservation it is recommended to freeze about 20 shoot tips (Photo1a). This step is carried out under sterile condition using a laminar chamber hood.
2) After one week of culture, the apical shoot tips (1 mm in size) are excited under a stereo microscope (Photo1b). This step is carried out under sterile condition using a laminar chamber hood.

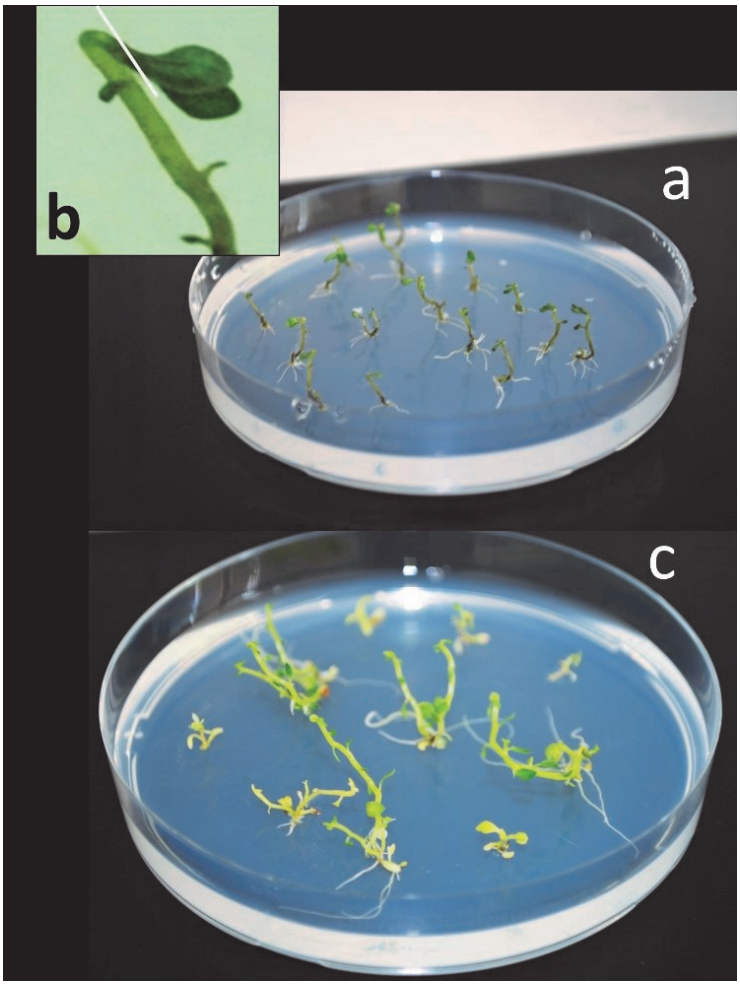

Photo 1. a) –7-day-old *in vitro* plants for shoot tips collection;
b) – 1 mm apical shoot tips; c) –regenerated plants

3) Shoot tips are incubated overnight on MS medium with 10.3% sucrose.
4) Shoot tips are transferred into 1.5 ml cryo-tubes with LM solution of 13.7% sucrose and 18.4% glycerol in MS medium for 20 min. The cryo-tubes are signed with a marker (name of the genotype, date of freezing).
5) In the next step, LM medium is removed and PVS2 solution is added into the same 1.5 ml cryo-tube. Shoot tips are incubated in PVS2 solution with 13.7% sucrose, 30% glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) in MS medium for 30 min.
6) In every cryo-tubes 10 shoot tips are placed and put directly in small dewar with LN. About 5-6 cryo-tubes (50-60 shoot tips) per genotype are frozen. The collected cryo-tubes are placed into plastic boxes and transferred into LN tanks for long-term storage.

7) Regeneration. Shoot tips from LN are rewarmed by immersion of the tubes into a water bath at 38°C for 2 min and washed three times with MS solution with high sucrose concentration (41.8%) and transferred on MSTo medium with 0.8% agar. After 6-8 weeks shoot tips are regenerated (Photo 1c).

Cryopreservation of potato pollen

1) Fresh or refrigerated pollen grains of certain genotypes are placed in cryo-tubes in non-sterile conditions.
2) Cryo-tubes with pollen are directly immersed in LN (Photo 2).
3) Regeneration. Pollen from LN is rewarmed during 1 h at room temperature and it is ready for usage.

Photo 2. Preservation of potato pollen in LN

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