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CRYOPRESERVATION

Development of a common PVS2 vitrification method for cryopreservation of several fruit and vegetable crops

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Abstract Many cryopreservation techniques are currently available, and it is common for new modifications to be developed for individual crops or specific genotypes. In this study, results of variations of the PVS2 cryopreservation protocol are compared to provide evidence for the suitability of a standard form of this technique for cryopreservation of a range of fruit, berry crops, and potato. Shoot cultures of *Malus*, *Solanum*, *Lonicera*, and *Berberis* were tested with variations of cold acclimation, pretreatment media, and PVS2 exposure times. A general protocol with some modifications was produced that was suitable for all four genera. The regenerative capacity of shoot tips after cryopreservation by this method exceeded a mean of 50% for *Malus*, *Solanum*, *Lonicera*, and *Berberis*, which is sufficient for setting storage in a cryobank. After liquid nitrogen storage, the shoot cultures that survived had a healthy appearance and developed rapidly. For each species tested, the only optimization required was the preparation of donor plants by cold acclimation and pretreatment. The choice of one common method simplifies the methodology for conducting experiments and storing a range of germplasm. The use of the PVS2 vitrification method with a 0.3-M sucrose pretreatment is multiuse and can be recommended as the most effective method for the cryopreservation of shoot tips from many plant species.

Keywords Apple · Honeysuckle · Barberry · Potato · PVS2-vitrification

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Introduction

Cryopreservation methods for cell cultures, tissues, buds, pollen, and embryos of plants are currently well developed and often successfully used in agriculture and scientific experiments. Deep freezing in liquid nitrogen (LN) at a temperature of − 196°C is one of the most promising ways to preserve vegetatively propagated plants, non-orthodox seed species, rare and endangered species, and various biotechnology products. This method of preserving retains the viability and regenerative capacity of the material indefinitely by exposure to LN (Lynch *et al.* 2007; Keller *et al.* 2008; Engelmann 2011).

Cryopreservation difficulties are associated with the peculiarities of plant cells having large size, large vacuoles, and high water content (Chang and Reed 1999; Wu *et al.* 1999; Lynch *et al.* 2007; Kaczmarczyk *et al.* 2008; Sakai *et al.* 2008; Engelmann 2011). Cell death can occur due to the formation of ice during the freezing process. Intracellular ice crystals cause mechanical damage during rapid freezing, which results in cell dehydration. In order to solve these problems, two main cryoprotection strategies for plant tissues are commonly used, which includes treatment of tissues by cryoprotectants (chemical substances that protect cells from exposure to low temperatures), or the removal of excess water from the cells that are capable of freezing (dehydrating). Both approaches have the same goal to reduce or prevent the formation of ice in the tissues. These goals are accomplished by several cryopreservation methods such as vitrification, encapsulation-dehydration, slow freezing, and droplet freezing that allow vitrification of the cell cytoplasm (Wu *et al.* 1999; Reed *et al.* 2001; Sakai *et al.* 2008; Tsai *et al.* 2009; Li *et al.* 2014).

Previously, cryopreservation protocols were optimized for four varieties and one wild form of apple using methods such as vitrification, encapsulation-dehydration, and slow freezing (Kushnarenko *et al.* 2009). It is important to note that in this

2009 study, the number of accessions was limited; however, the results allowed for further research not only on apple (*Malus* spp.), but also various other plant species. As this study indicated, PVS2 vitrification proved to be an optimal cryopreservation method for apple, but also for potato (*Solanum tuberosum*), which a later study demonstrated (Kushnarenko *et al.* 2015). Although there are many individual techniques that have been optimized for individual genotypes, this type of individual optimization is time consuming and delays the implementation of cryopreserved storage. The use of a common cryopreservation technique would greatly aid the addition of cryopreserved samples to cryobanks at genetic resources centers (Reed *et al.* 2003; Reed 2008).

Toward this end, studies were conducted to develop a general PVS2 cryopreservation protocol for certain fruit, berry crops, and potato. In this study, research was conducted using a significant number of accessions. Different varieties of apple and potato from Kazakhstan and foreign selections were used, in addition to wild forms of *Malus sieversii* from several regions of Ile-Alatau National Park of Kazakhstan. This study cites cryopreservation results from several wild forms and varieties of honeysuckle (*Lonicera* spp.) and barberry (*Berberis* ssp.) such as *L. iliensis* and *B. iliensis*, which remain in the Red Book of Kazakhstan (http://www.redbookkz.info/). The novelty of this research is the fact that this successful attempt at their cryopreservation is the first in the world. In this study, these results are compared and recommendations are made for a general technique to implement storage of shoot cultures in a cryobank.

Materials and Methods

Plant material

Malus Thirty-six apple accessions from farmers' orchards and the Kazakh Research Institute of Horticulture and Viticulture were included: 15 recognized and promising cultivars, and 9 clonal apple rootstocks (*M. domestica* Borkh.) of Kazakh and foreign breeding (cultivars: Aport Alexander, Aport Alexander form 5, Aport krovavo-krasnyi, Aport krovavo-krasnyi form 1, Voskhod, Golden Delicious, Gold Rush, Grushovka Vernenskaya, Egemen, Zarya Alatau, Maksat, Renet Landsbergskyi, Royal Red Delicious, Sinap Almatinskyi, Suyslepper; clonal rootstocks: arm 18, arm 18 form 1, B 16-20, Zhetysu 5, M 9, MM 106, MM 106 form 1, 62-64 396 form 1, 62-396 form 2; and 12 forms from Ile-Alatau National Park (KG, KG1, KG4, KG5, KG7, KG8, KG9, KG10, KG13, KG19, KG20, TM-6) of wild apple (*M. sieversii* (Ledeb.) M. Roem).

Solanum Twelve cultivars and three hybrids of potato (*Solanum tuberosum* L.) from the Kazakh Research Institute of Potato and Vegetable Growing and foreign breeding were studied (cultivars: Astana, Aul, Zholbarys, Karasaiskyi, Nartau, Nikitka, Novinka, Nerli, Soyuz, Tamyr, Khozyayushka, Shagalaly; hybrids: 12-04-01, 18-04-01, 21-09-02).

Lonicera Four accessions of wild honeysuckle species from the river banks of the Ili and the Altai region of Kazakhstan were studied: three populations of *L. iliensis* Pojark, one population of *L. altaica* Pall., and one cultivar—Galochka *L. altaica* from the Altai Botanical Garden collection.

Berberis Six accessions of wild barberry: two forms of *B. iliensis* M. Pop from the river banks of the Ili, two forms of *B. heteropoda* Schrenk from the Ile-Alatau National Park, two forms of *B. integerrima* Bunge from Sairam-Ugamsky National Park of Kazakhstan and Zaravshan Valley Tugai Reserve of Uzbekistan; one cultivar: Superba Ottawa barberry from the Almaty Forest Nursery.

Micropropagation All cultures were grown in glass culture vessels (237 mL) (Phytotechnology Laboratories®, Lenexa, KS) in a growth room at 24°C with 16-h photoperiod of white fluorescent lights at 40 μM m^{-2} s^{-1} (standard growth room conditions) with two types of OPPLE tubular fluorescent lamps: YK 21RR 16/G 21W 6500K RGB and YK 21RL 16/G 21W 4000K RGB supplied by ElectroComplex in Corporation, Almaty, Republic of Kazakhstan (http://elcor.kz). Subcultures were completed at 3–4-wk intervals. All shoots were propagated on Murashige and Skoog nutrient medium (MS) (Murashige and Skoog 1962) containing 30 g L^{-1} sucrose. The medium was adjusted to pH 5.7 with 0.1 N NaOH and autoclaved at 121°C for 20 min. Plant growth regulators and supplements listed below for each culture. All chemicals were supplied by SPF Mediland, Almaty, Republic of Kazakhstan (http://mediland.kz) unless otherwise specified.

In vitro apple shoots were cultured on MS-based medium described above with the addition of 2.2 μM N^6-benzylaminopurine (BAP), 0.04 μM indole-3-butyric acid (IBA), 1.75 g L^{-1} Gelrite™ (Phytotechnology Laboratories®), and 4 g L^{-1} Plant TC agar (Phytotechnology Laboratories®), pH 5.7 (Romadanova *et al.* 2015). *In vitro* aseptic potato plants were obtained from tuberous material (Kushnarenko *et al.* 2013). The propagation of *in vitro* shoots was carried out on MS-based medium with 30 g L^{-1} sucrose, 2 mg L^{-1} calcium D-pantothenate (PC) (Applichem, Darmstadt, Germany), 1.75 g L^{-1} Gelrite™ (Phytotechnology Laboratories®), 3 g L^{-1} Plant TC agar (Phytotechnology Laboratories®), and pH 5.7. The propagation of honeysuckle shoots was performed on MS-based medium with 4.44 μM BAP, 1.75 g L^{-1} Gelrite™, and 4 g L^{-1} Plant TC agar. Barberry shoots were micropropagated on MS-based medium with 7.0 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 9.3 mg L^{-1} $Na_2EDTA \cdot$

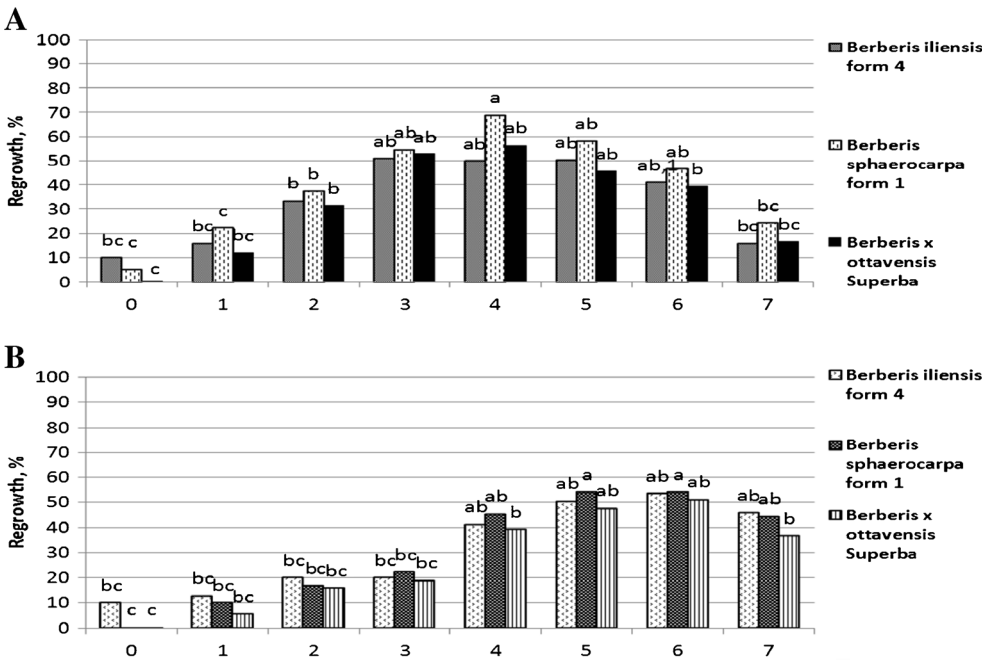

Figure 1. Effect of cold acclimation for 0 to 7 wk on regrowth of barberry shoot tips after cryopreservation. Loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min. (*A*) Cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; (*B*) cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light. Means indicated by different *letters* differ significantly at $p \leq 0.05$.

$2H_2O$, 166 мг/л $CaCl_2$, 3.55 μM BAP, 0.29 μM gibberellic acid, 0.08 μM IBA, 1 mg L^{-1} ascorbic acid, 2 mg L^{-1} PC, 1.75 g L^{-1} Gelrite™, and 4 g L^{-1} Plant TC agar, pH 5.7 (Romadanova *et al.* 2016).

Cold acclimation of shoot cultures Shoots of apple, honeysuckle, and barberry in glass culture vessels (237 mL) (Phytotechnology Laboratories®) on the nutrient medium described for each culture were acclimated in two modes: (1) variable temperatures (Chang and Reed 2000): 8 h at 22°C, light intensity 10 μM m^{-2} s^{-1} and 16 h at − 1°C in the dark in climatic chamber (Lab-line Environette, Conroe, TX); (2) constant temperature of 4°C: 8 h, light intensity 10 μM m^{-2} s^{-1}, 16 h in the dark. The duration of cold acclimation (CA) ranged from 1 to 7 wk. *In vitro* shoots that were not cold treated served as the control. Shoot tips (shoot apices

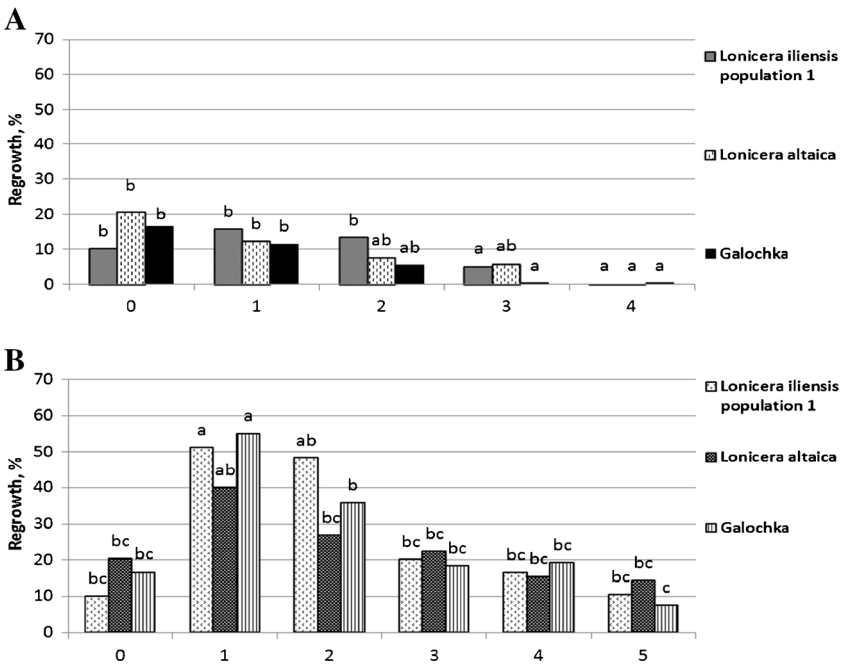

Figure 2. Effect of cold acclimation from 0 to 5 wk on regrowth of honeysuckle shoot tips after cryopreservation. (*A*) Cold acclimation of donor plants at − 1°C, 16 and + 22°C, 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min; (*B*) Cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light; PVS2 treatment at 0 °C for 80 min. Means indicated by different *letters* differ significantly from each other at $p \leq 0.05$.

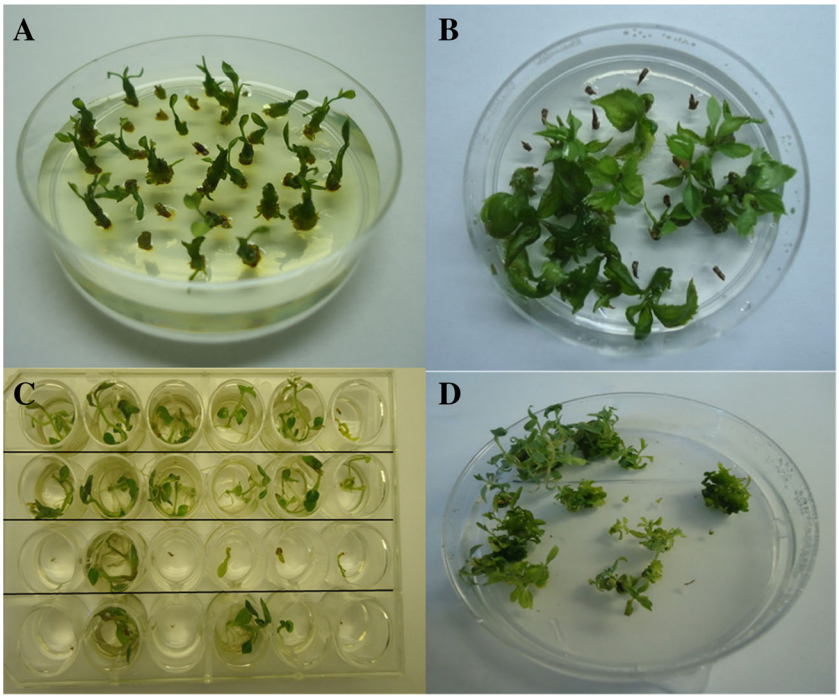

Figure 3. Regrowth of shoot tips over 3 wk after cryopreservation. (*A*) *Berberis* × *ottawensis* Ottawa Superba—4-wk cold acclimation of donor plants at − 1°C, 16 h, and + 22°C 8 h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min; (*B*) Wild apple form KG9—3-wk cold acclimation of donor plants at − 1°C, 16 h, and + 22°C 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min; (*C*) Potato, Nerli cultivar—1-d preculture of shoot tips on a medium containing 0.3 M sucrose at 24°C, 16-h dark, and 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min (Kushnarenko *et al.* 2015); (*D*) Honeysuckle, Galochka cultivar—1-wk cold acclimation of donor plants at 4°C, 16 h, and 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at 4°C, 16 h, and 8-h light; PVS2 treatment at 0°C for 80 min.

with 3–4 leaf primordia 0.8–2.0 mm) were aseptically isolated from CA *in vitro* shoots for the experimentation. Potato shoot tips were dissected from non-acclimated shoots because it was previously shown that CA is not required for potato (Kushnarenko *et al.* 2015).

PVS2 vitrification The general PVS2 vitrification protocol was performed as noted below based on Sakai *et al.* (1991, 2008).

Preculture Shoot tips from CA shoots (apple, barberry, and honeysuckle) and non-CA shoots of potato were excised and cultured in Petri dishes (60 mm × 15 mm) (Phytotechnology Laboratories®) on MS-based medium with 30 g L^{-1} sucrose, 5% (*v*/*v*) dimethylsulfoxide (DMSO), 3.5 g L^{-1} Plant TC agar, and 1.75 g L^{-1} Gelrite™, at pH 5.7. Apple and barberry were precultured for 2 d at − 1°C 16-h dark and + 22°C 8-h light; honeysuckle was precultured for 2 d at 4°C 16-h dark and 8-h light; and potato was precultured for 1 d at 24°C with 16-h dark and 8-h light. Alternative treatments were on MS medium with 0.3 M sucrose for 1 or 2 d at either variable temperatures of 22°C in 8-h light and − 1°C in 16-h dark (22°C/ − 1°C), or at a constant temperature of 4 or 24°C with 16-h dark and 8-h light (controls).

Loading Shoot tips were placed in 1.2-mL cryovials (Corning®, Corning, NY) (20 shoot tips per cryovial) containing loading solution of 2 M glycerol and 0.4 M sucrose for 20 min at 24°C for potato, or 0°C for apple, barberry, and honeysuckle, or in some experiments, this stage was excluded.

Cryoprotectant treatment Shoot tips (in cryovials) were exposed to PVS2 cryoprotectant (30% (*v*/*v*) glycerol, 15% (*v*/*v*) ethylene glycol, and 15% (*v*/*v*) dimethylsulfoxide (DMSO) in liquid MS medium containing 0.4 M sucrose adjusted to pH 5.7) for 20, 30, 40, 60, and 80 min at a temperature of 24°C for potato, or 0°C for apple, barberry, and honeysuckle.

Liquid nitrogen exposure to regrowth conditions After PVS2 treatment, cryovials were immersed in liquid nitrogen (LN) for 15–20 min. Vials were rewarmed in a 45°C water bath for 1 min and then in 25°C water for 1 min. Shoot tips were immediately rinsed twice with liquid MS medium containing 1.2 M sucrose and placed on MS multiplication

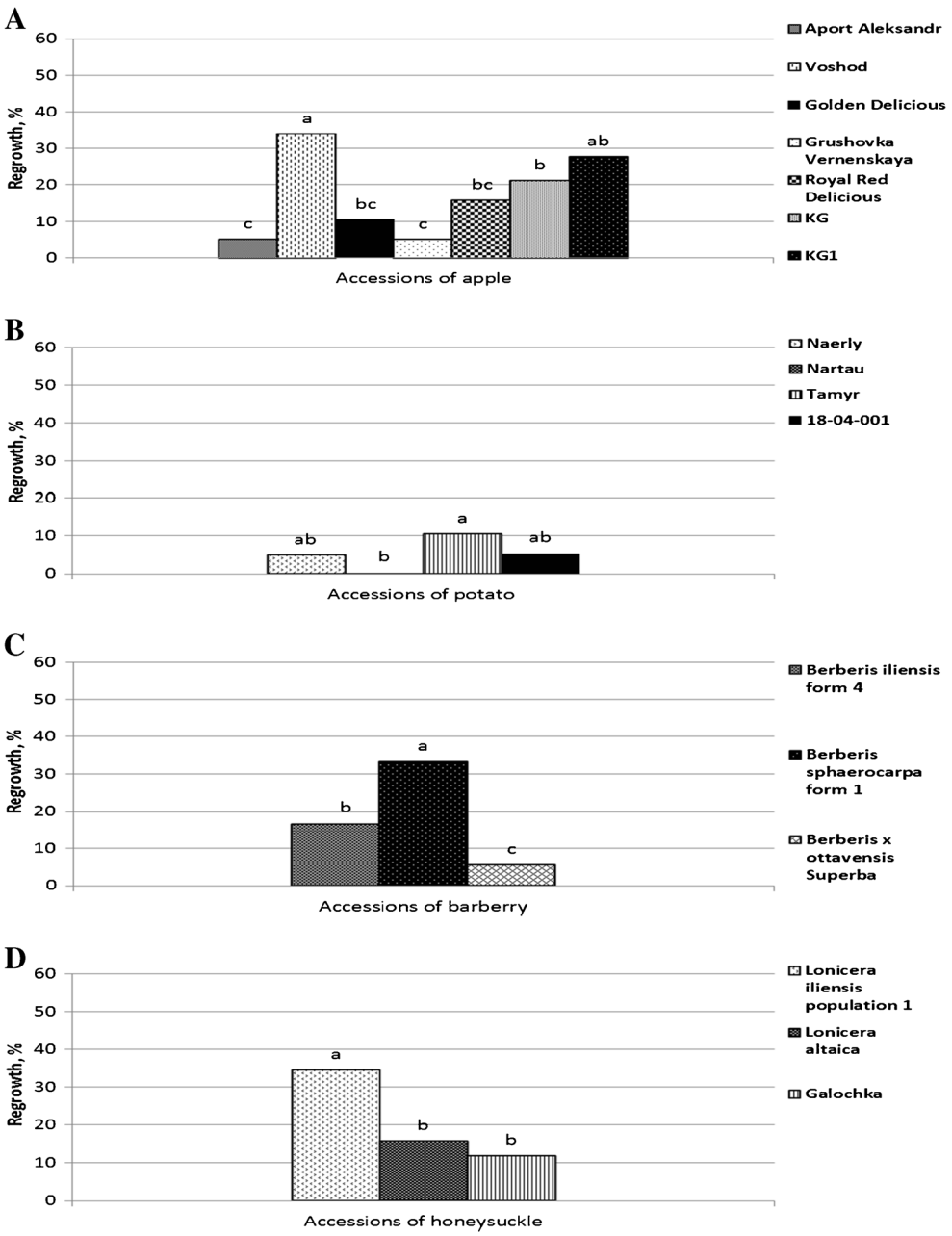

Figure 4. Effect of preculture of shoot tips (MS with 5% (*v*/*v*) DMSO) on regrowth of cryopreserved shoot tips. (*A*) Apple—3-wk cold acclimation of donor plants at − 1°C, 16 h, and + 22°C 8-h light; 2-d preculture of shoot tips on MS with 5% (*v*/*v*) DMSO at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min; (*B*) Potato—1-d preculture of shoot tips on MS with 5% (*v*/*v*) DMSO at 24°C 16-h dark/8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min; (*C*) Barberry—4-wk cold acclimation of donor plants at − 1°C, 16 h, and + 22°C 8-h light; 2-d preculture of shoot tips on MS with 5% (*v*/*v*) DMSO at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min and (*D*) Honeysuckle—1-wk cold acclimation of donor plants at 4°C, 16 h, and 8-h light; 2-d preculture of shoot tips on MS with 5% DMSO at 4°C, 16 h, and 8 h.

medium (the same composition as described above for each culture) for regrowth under standard conditions. Shoot growth for potato shoot tips was performed after cryopreservation for 1 wk in the dark (covered shoot tips) and then transfer to standard growth conditions.

Experimental design Shoot cultures of each genotype were micropropagated as indicated above. Optimization of the vitrification procedure was performed at the four following four stages: cold acclimation (duration and temperature) for honeysuckle and barberry shoots; preculture of shoot tips (MS with 5% (*v*/*v*) DMSO or 0.3 M sucrose); shoot tips loading in 2 M glycerol with 0.4 M sucrose (effect of this stage); and PVS2 treatment (duration). For each technique and genotype, 20 shoot tips were used for each treatment. The experiments were performed in three replications, $n = 60$. Statistical analysis of the experimental data was conducted according to standard procedures described in the manual by Lakin 1990 and the SYSTAT version 12.0 software package (Systat Software Inc., San Jose, CA). All data presented as percentages ± standard error were subjected to arcsine transformation before the statistical analysis. ANOVA using SAS, version 9.2, for windows (SAS Institute Inc. Cary, NC) was applied to analyze the data.

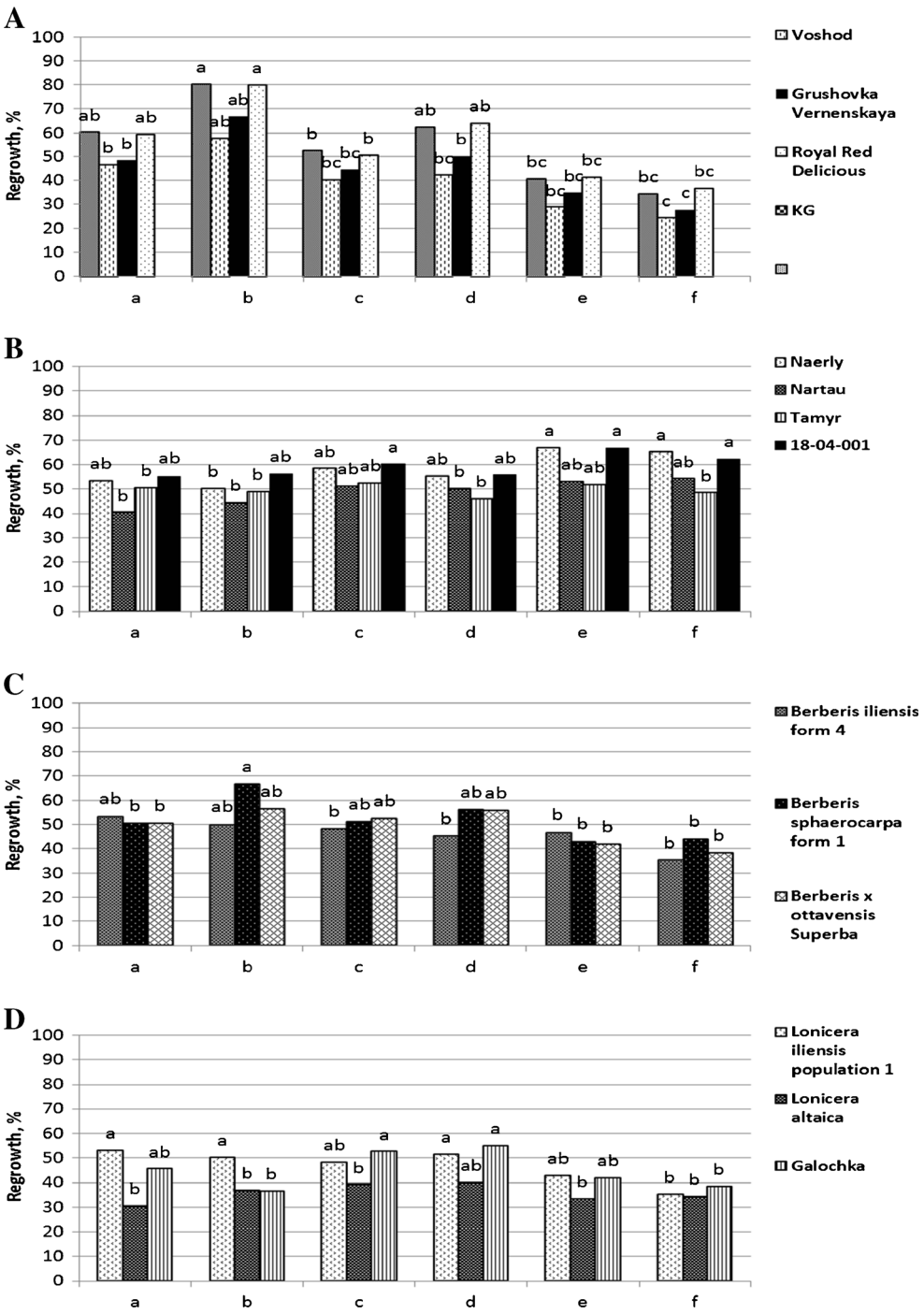

Figure 5. Influence of temperature and duration of shoot tips precultured on a medium containing 0.3 M sucrose, 16-h dark, and 8-h light on regrowth of cryopreserved shoot tips: (*a*) 1 d at − 1°C, 16-h dark, and + 22°C 8-h light; (*b*) 2 d at − 1°C, 16-h dark, + 22°C 8-h light; (*c*) 1 d at 4°C; (*d*) 2 d at 4°C; (*e*) 1 d at 24°C; (*f*) 2 d at 24°C. (*A*) Apple—3-wk. cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min; (*B*) Potato—1-d shoot tips preculture on a medium containing 0.3 M sucrose at 24°C, 16-h dark, and 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min (Kushnarenko *et al.* 2015); (*C*) Barberry—4-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min; (*D*) Honeysuckle—1-wk cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light; PVS2 treatment at 0°C for 80 min. Means indicated by different letters differ significantly from each other at $p \leq 0.05$.

Results and Discussion

In the current study, there were a few modifications of the PVS2 vitrification protocol needed among the genera tested, but accessions within each genus could be stored with one procedure. To create the PVS2 protocol for vitrification, several steps were optimized for each culture including CA of the donor plants, shoot tip precultures, loading, and the cryoprotectant treatment. For successful cryopreservation, many scientists use CA shoots, in which the duration and temperature regimes vary for different cultures (Chang and Reed 1999; Chang and Reed 2000; Kryszczuk *et al.* 2006; Kaczmarczyk *et al.* 2008). Apple shoots of some cultivars were successfully CA (Kushnarenko *et al.* 2009). In the current study, apple shoots required a CA at 22°C/− 1°C for 3 wk, which resulted in a regrowth success of 50–96.3%. Barberry

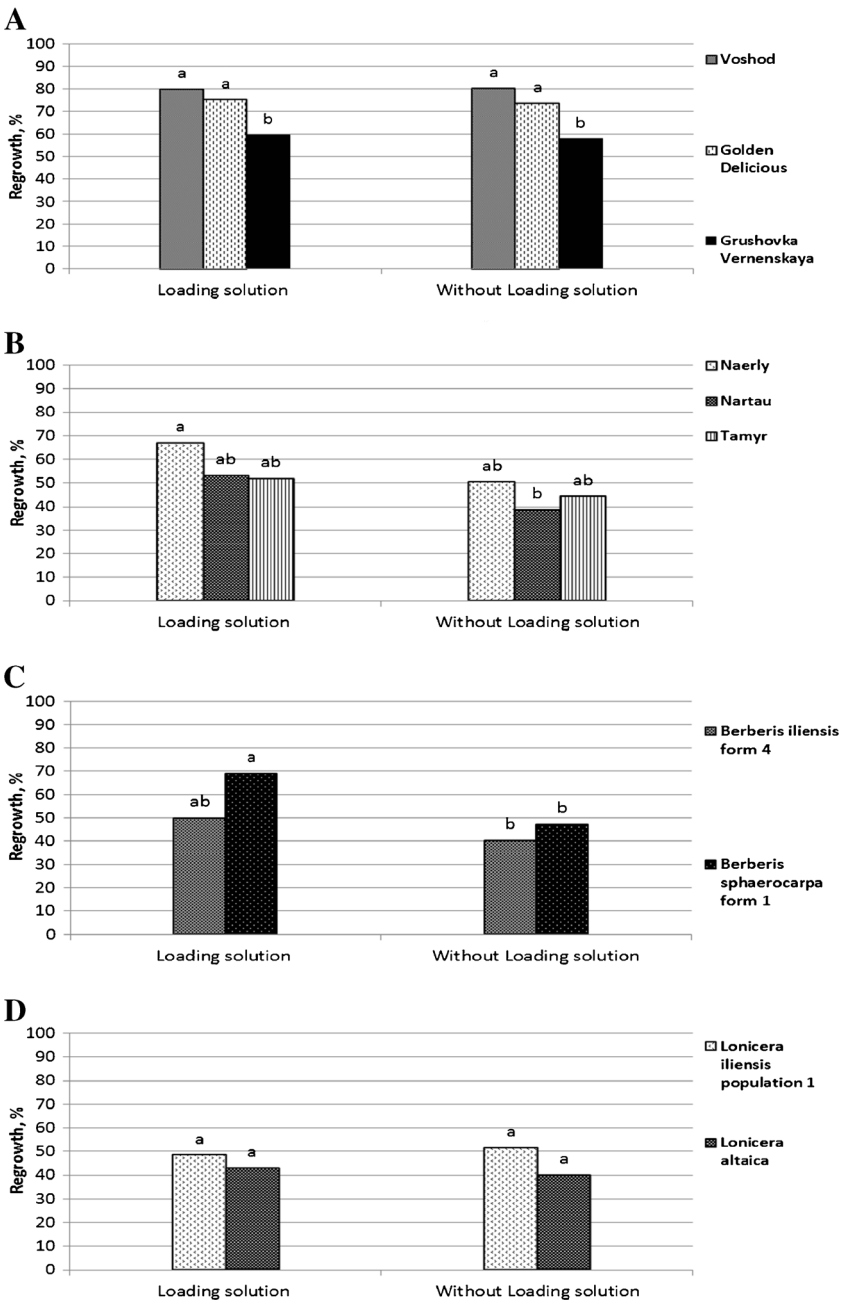

Figure 6. Effect of treatment in loading solution of 2 M glycerol solution with 0.4 M sucrose for 20 min on the regrowth of cryopreserved shoot tips. (*A*) Apple—3-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min (Kushnarenko *et al.* 2009); (*B*) Potato—1-d shoot tips preculture on a medium containing 0.3 M sucrose at 24°C, 16-h dark, and 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min (Kushnarenko *et al.* 2015); (*C*) Barberry—4-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min; (*D*) Honeysuckle—1 wk cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light; PVS2 treatment at 0°C for 80 min. Means indicated by different letters differ significantly from each other at $p \leq 0.05$.

required a CA at 22°C/− 1°C for 3–5 wk that resulted in a 50–70% and 4–7 wk CA at 4°C for a 40–50% regrowth (Fig. 1). For CA of honeysuckle shoots 1 wk at 4°C was optimal and resulted in a 40–55% regrowth rate compared to poor recovery with 22°C/− 1°C (Fig. 2). CA of the donor plants was not required for potato (Kushnarenko *et al.* 2015). These results differ from those obtained by Kaczmarczyk *et al.* (2008) for CA of donor potato plants, but are similar to the results of Kryszczuk *et al.* (2006).

Natural and climatic growth conditions of the crops studied determine the duration and temperature mode of their hardening (Fig. 3). Thus, a cold acclimation is not required for thermophilic potato. For most plants of temperate climate, CA is required (Sakai and Larcher 1987; Reed 1988). Apple, honeysuckle, and barberry grow at high altitudes, where sharp temperature changes can occur in the span of a day. The difference between day and night temperatures can be over 30°C. Experiments showed that hardening is necessary for these crops. The duration and temperature mode of hardening depends on the natural resistance to cold of the genotypes studied, as previously shown for apple (Kushnarenko *et al.* 2009).

Pretreatment with either DMSO or sucrose is often used to improve the regrowth of shoot tips following cryopreservation

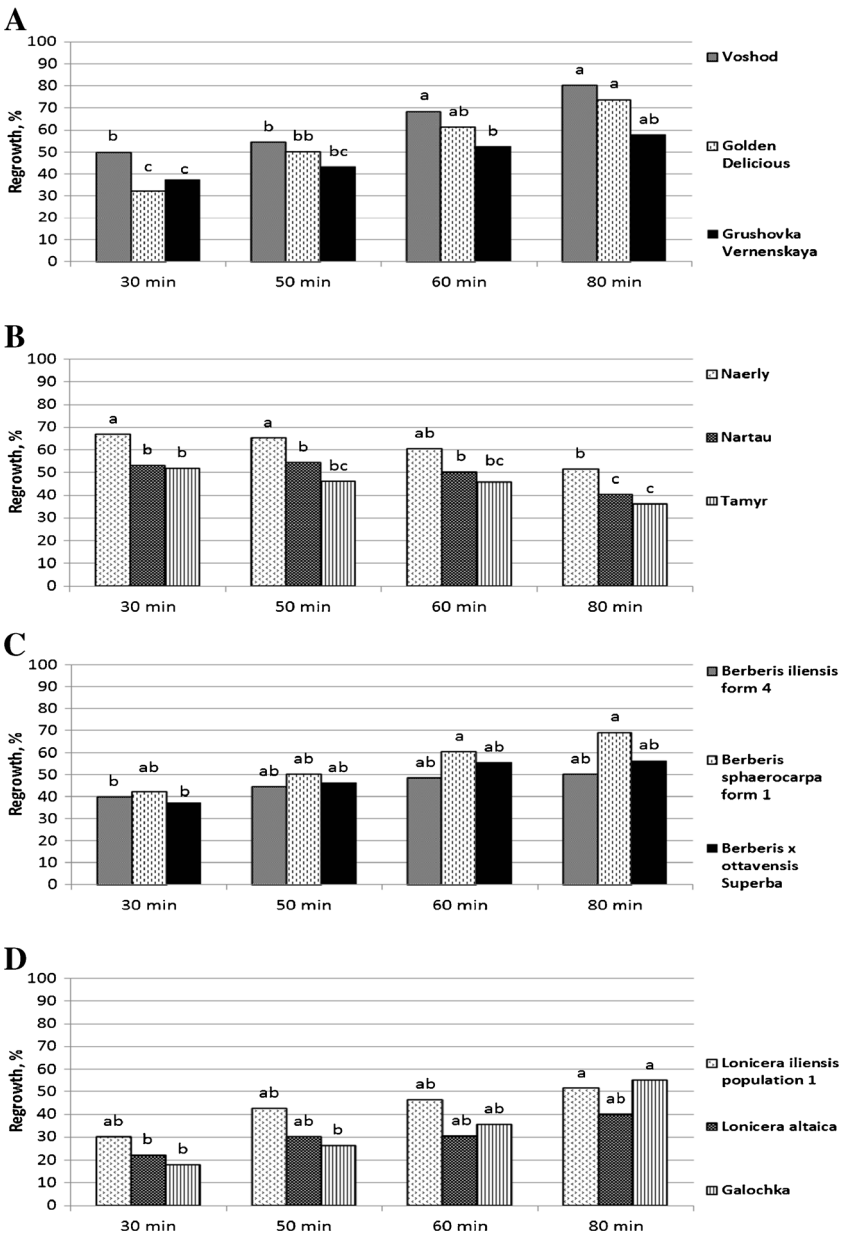

Figure 7. Effect of treatment duration in PVS2 solution at 0°C for apple, barberry, honeysuckle and at 24°C for potato on the regrowth of shoot tips. (*A*) Apple—3-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min; (*B*) Potato—1-d shoot tips preculture on a medium containing 0.3 M sucrose at 24°C, 16-h dark, and 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min (Kushnarenko *et al.* 2015); (*C* Barberry—4-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min; (*D*) Honeysuckle—1-wk cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light; PVS2 treatment at 0°C for 80 min. Means indicated by different letters differ significantly to each other at $p \leq 0.05$.

(Reed 2008). Many studies have shown a positive effect of 5% (*v*/*v*) DMSO on the shoot growth after cryopreservation (Reed *et al.* 2001). However, this study did not reveal promising regenerative results with 5% (*v*/*v*) DMSO. In some cases, DMSO had a toxic effect on cells, and in other cases, it is not effective for some genotypes. For the genera of the present study, the regrowth percentage for all accessions after cryopreservation of shoot tips precultured on a medium containing 5% (*v*/*v*) DMSO did not exceed 34%, the development was delayed, and shoot tips were often yellow (Fig. 4). Therefore, it was decided to continue the experiments with a medium of 0.3 M sucrose for the precultivation of meristems.

Exposure of shoot tips on MS medium with 0.3 M sucrose was optimal for all of the plants studied, and 2 d of preculture were needed for apple, honeysuckle, and barberry, but only 1 d was required for potato. At this stage, variable temperatures of 22°C/− 1°C were optimal for apple and barberry, 4°C for honeysuckle, and 24°C for potato (Figs. 3 and 5).

Exposure of apical meristems to a loading solution of 2 M glycerol with 0.4 M sucrose had a positive impact on the regrowth of apical meristems of barberry and potato after cryopreservation, but had no significant impact on the regrowth of apple and honeysuckle shoots (Fig. 6). A treatment with PVS2 cryoprotectant revealed a positive effect for all accessions at different durations of exposure and at different temperatures. Thus, regrowth percentages increased significantly with longer exposure to PVS2 at 0°C for apple and

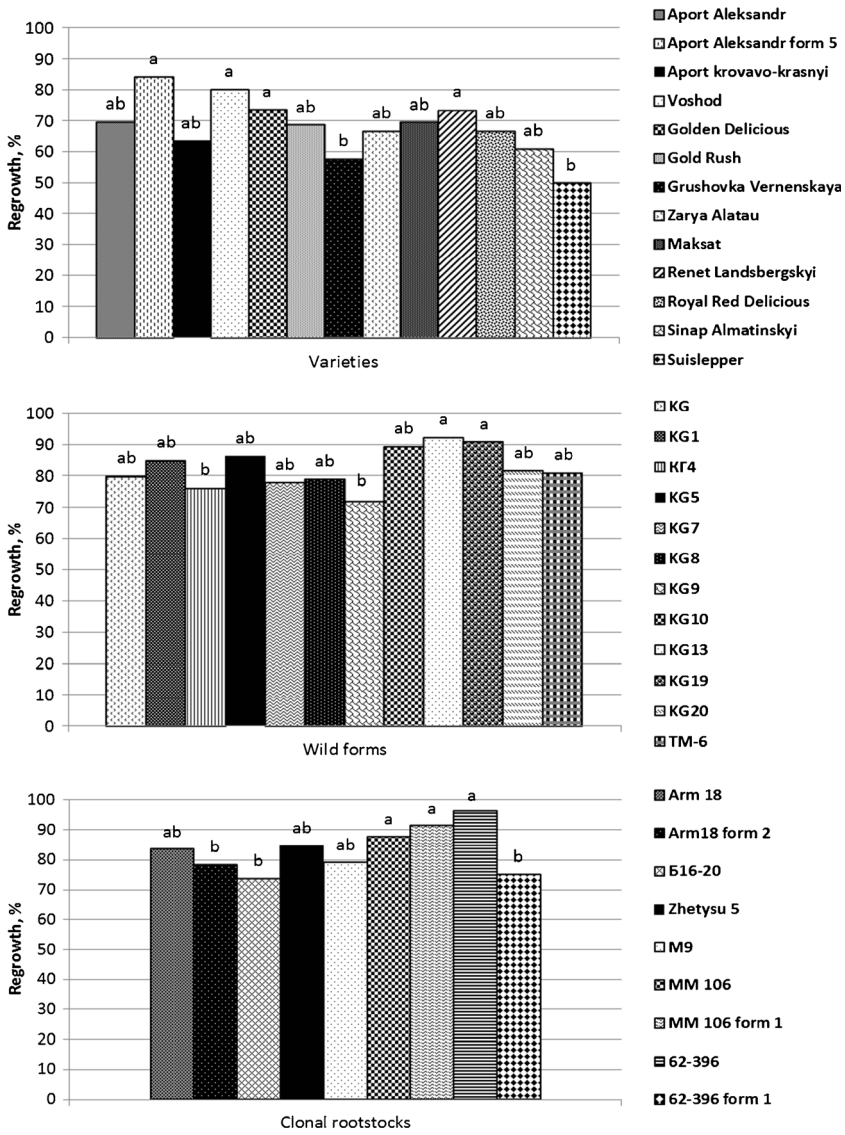

Figure 8. Regrowth of *Malus domestica* Borkh and *Malus sieversii* (Ledeb) M. Roem from shoot tips after cryopreservation by PVS2 vitrification—3-wk cold acclimation of donor plants at − 1°C, 16 h, and + 22°C 8-h light; 2-d preculture of shoot tips on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; PVS2 treatment at 0°C for 80 min. Means indicated by different *letters* differ significantly to each other at $p \leq 0.05$.

honeysuckle, but were not significantly different (NSD) for barberry (Fig. 7). For potato, 30 min of PVS2 exposure was significantly better at 24–25°C than the 80-min treatment (Fig. 7*B*). Treatment of barberry shoot tips with PVS2 cryoprotectant for all of the treatment times were NSD, and the duration of the experiment could be decreased using the shorter times (Fig. 7*C*). Honeysuckle treatments at 60 and 80 min were also NSD (Fig. 7*D*).

These experiments revealed that the PVS2 vitrification method provides adequate regrowth for apple, potato, honeysuckle, and barberry with only slight variations in the CA, preculture, and PVS2 treatment. Thus, in *Malus*, the regrowth percentage of cultivars ranged from 60 to 84.6%; for clonal rootstocks from 72.1 to 95.4%; and from 71.6 to 92.1% for wild forms (Fig. 8). Regrowth of clonal rootstocks and wild forms is slightly higher than the cultivars, perhaps due to the natural hardiness of these accessions (Kushnarenko *et al.* 2009). After cryopreservation, regrowth of potato was in the range of 40 to 67.6% (Fig. 9). Differences in the regrowth percentages were not identified among Kazakh and foreign cultivars, and almost all of the results were within the limits of error. There was no difference in the regrowth percentage after cryopreservation between wild species of barberry and cultivar Ottawa Superba, and in wild species of honeysuckle and cultivar Galochka. Regrowth ranged from 50.0 to 82.1% for barberry, and from 40.0 to 62.3% for honeysuckle (Fig. 9).

A comparison of PVS2 vitrification modifications on four genera of micropropagated shoots indicated some differences and similarities in the cryopreservation procedure. Each group was successfully cryopreserved with > 50% regrowth using this technique with some variations (Fig. 10). This result is similar to the use of a standard

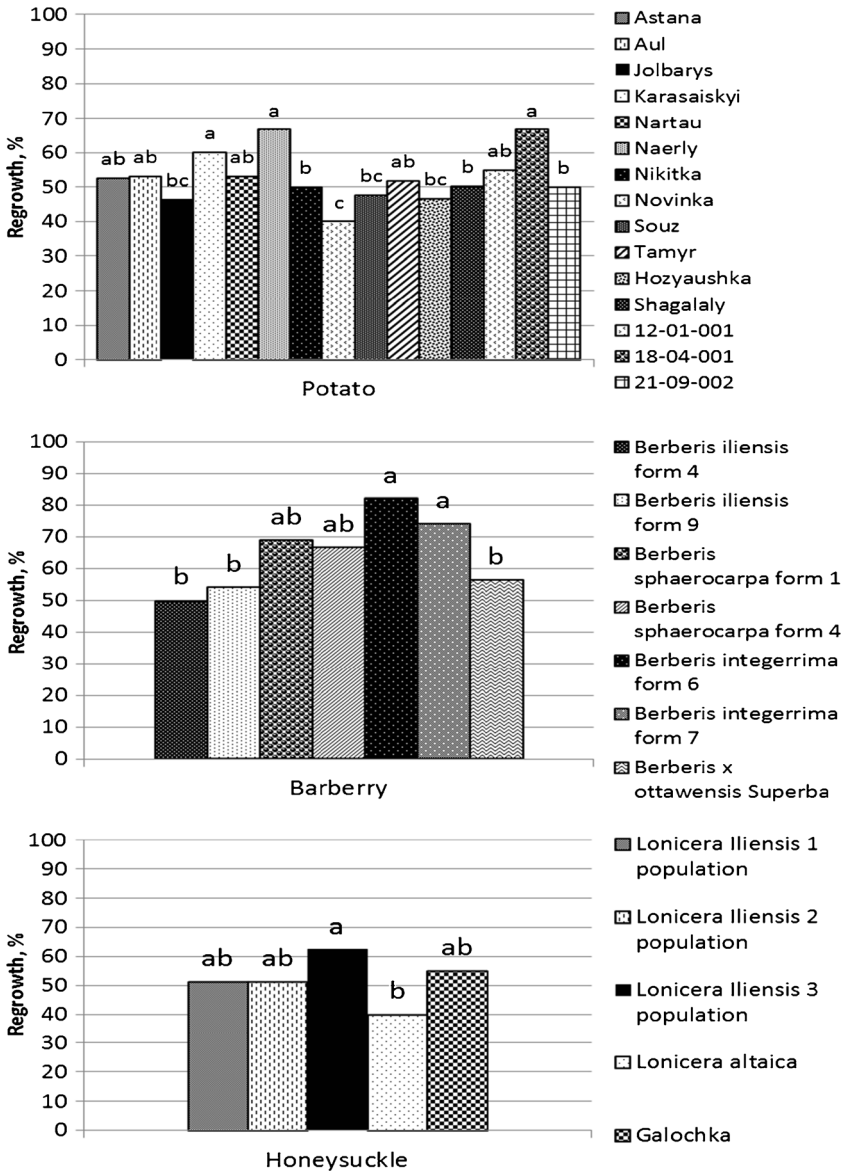

Figure 9. Regrowth (from shoot tips after cryopreservation by PVS2 vitrification): potato—1-d shoot tips preculture on a medium containing 0.3 M sucrose at 24°C, 16-h dark/8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 24°C for 20 min; PVS2 treatment at 24°C for 30 min; barberry—4-wk cold acclimation of donor plants at − 1°C, 16-h dark, and + 22°C 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at − 1°C, 16-h dark, and + 22°C 8-h light; loading in 2 M glycerol solution with 0.4 M sucrose at 0°C for 20 min; PVS2 treatment at 0°C for 80 min; honeysuckle—1-wk cold acclimation of donor plants at 4°C, 16-h dark, and 8-h light; 2-d shoot tips preculture on a medium containing 0.3 M sucrose at 4°C, 16-h dark, and 8-h light; PVS2 treatment at 0°C for 80 min. Means indicated by different *letters* differ significantly from each other at $p \leq 0.05$.

slow-freezing cryopreservation procedure developed for *Pyrus* and *Humulus* shoot tips, and the mean regrowth was 54% for a large group of diverse genotypes (Reed *et al.* 2003).

CA of shoots, preculture of shoot tips, shoot tip loading, and PVS2 treatment are necessary for successful cryopreservation of the tested samples. A slight difference in the duration and temperature regimes of the CA is due to the genetic stability of the test samples to temperature changes. The genotypes studied did not show a high percentage of regeneration in shoot tips that were cultivated on 5% (*v*/*v*) DMSO after cryopreservation. Perhaps some modifications could lead to positive results; however, because precultivation on 0.3 M sucrose in all samples gave a high percentage of shoot tip regeneration after cryopreservation, the need for additional experiments is not necessary. Exposure of apical meristems to a loading solution of 2 M glycerol with 0.4 M sucrose had no significant impact on the regrowth of apple and honeysuckle shoots. Perhaps for these CA samples and precultured shoot tips on 0.3 M sucrose, it is already enough to get high regrowth results after cryopreservation. Nevertheless, for potato and barberry, this stage is critical for obtaining good regrowth results. The most important stage was PVS2 treatment, as it is necessary for all of the genotypes studied. For apple and berry crops, this stage is effective at 0°C, for thermophilic potatoes the effective temperature was 24°C. Exposure time in PVS2 for potatoes was 30 min, whereas for apple and berry, the time was 80 min. This is likely because the reaction rate of cryoprotector penetration into plant cells of positive temperatures is faster.

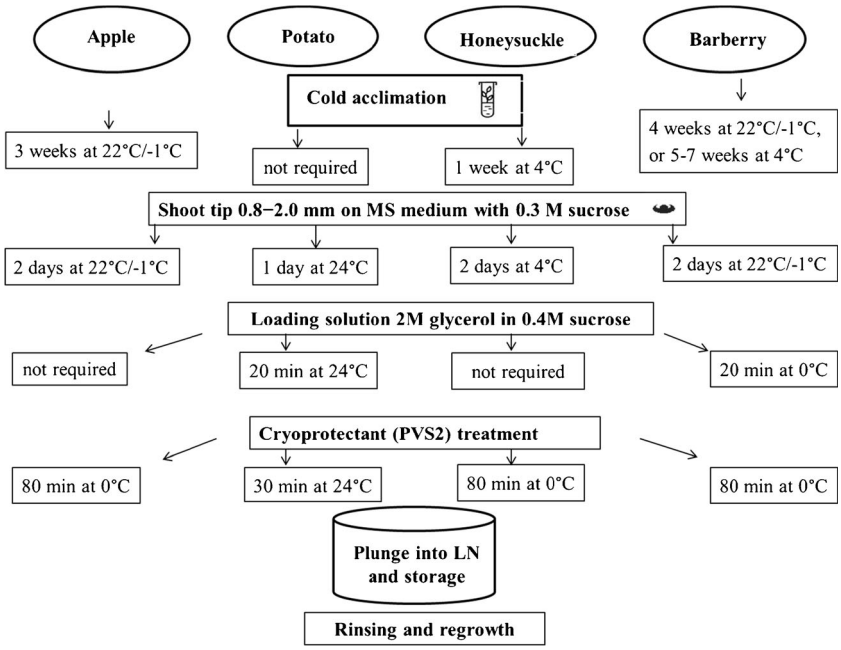

Figure 10. Stages of cryopreservation for apple, potato, honeysuckle, and barberry by PVS2 vitrification method.

Conclusions

The PVS2 vitrification method with a 0.3-M sucrose preculture treatment was shown to be suitable for cryopreservation for a range of fruit, berry crops, and potato and can be recommended as an effective method for the cryopreservation of shoot tips. The regenerative capacity of shoot tips after cryopreservation by this method exceeds a 50% average regrowth rate for all the studied cultures, and this is sufficient for creating a cryobank. After ultralow temperatures, shoot cultures had a bright-green color and developed quite rapidly. For each species, only a slight modification of the protocol was needed.

The choice of one common method simplifies the methodology for conducting experiments and eliminates the need to purchase additional reagents and expensive equipment used for other methods of cryopreservation. As a result of these studies, an aseptic disease-free collection of *in vitro* plants and shoot tips was created in the cryobank at the Institute of Plant Biology and Biotechnology. These cultures will be used to produce seedlings of a super elite class for nursery farms and will also be available for use in the breeding process to improve existing and future cultivars. *In vitro* plant material may be used for international exchange of plant resources and genetic material.

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