



Cryopreservation of highbush blueberry, strawberry, and saskatoon using V and D cryo-plate methods and monitoring of multiplication ability of regenerated shoots

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Abstract

The purpose of this work was to apply V and D cryo-plate methods for cryopreservation of *Vaccinium corymbosum* ‘Toro’, *Fragaria × ananassa* ‘Clery’, and *Amelanchier alnifolia* (Nutt.) M. Roem. and to monitor the multiplication capacity of shoots regenerated from cryopreserved explants. Shoot tips pre-cultured for 1 d at 23°C in the dark on medium containing 0.3 M sucrose were used as explants. Loading was performed in a solution containing 2 M glycerol and 0.8 M sucrose (30 min at room temperature). In the V cryo-plate, dehydration was carried out at room temperature (20 to 50 min) using the following plant vitrification solutions: original PVS2, 90% PVS2 solution, and PVS3. Regarding the D cryo-plate, dehydration was performed in closed glass containers over silica gel for 2, 2.5, or 3 h. In both protocols, rewarming was carried out in a 1.0 M sucrose solution (15 min at 25°C). Regenerated shoots were multiplied and multiplication parameters were monitored after the second subculture. Using the V cryo-plate method, the highest regrowth in highbush blueberry was obtained following 50-min treatment with all three VSs (61.7 to 80.9%). The D cryo-plate method was even more suitable with maximum regrowth of 89.4% achieved after 2.5 h of desiccation. For strawberry, 62.5% was the highest regrowth recorded using PVS3-based V cryo-plate method while 83.3% of regrowth was observed using D cryo-plate protocol. Regrowth of saskatoon reached a maximum of 50% after 50-min treatment with PVS3 while it did not exceed 40% in other treatments. By the second subculture, shoots regenerated from cryopreserved explants regained and even exceeded the multiplication capacity of shoots regenerated from non-cryopreserved explants. This study is the first to present the successful application of the V cryo-plate method in highbush blueberry, as well as the utilization of both V and D cryo-plate methods in saskatoon.

Keywords Highbush blueberry · Strawberry · Saskatoon · Vitrification solution · Desiccation

Introduction

There has always been a demand for alternative crops with unique nutritional traits. Small fruits, or “berries” for short, are small- to medium-sized fruits growing on perennial herbs, vines, or shrubs (Debnath 2003). Berries belong to

the group of economically important crops, mainly for being a valuable and natural source of many biologically active compounds, such as vitamins, antioxidants, flavonoids, and anthocyanins (Moyer *et al.* 2002; Castrejón *et al.* 2008). *Vaccinium* spp. and *Fragaria* spp. are among the berries known worldwide, while *Amelanchier alnifolia* (Nutt.) M. Roem. is grown regionally (Ochmian *et al.* 2013).

A. alnifolia (Nutt.) M. Roem. (saskatoon) is a fruit-bearing shrub native to the Northern Hemisphere that is attractive for its frost and disease resistance and decorative purposes. Its fruit quality is very similar to blueberries, and the ripe berries contain high levels of vitamins C and B₁₂ and essential minerals (Mazza 2005). *Vaccinium corymbosum* (highbush blueberry) is an economically important small fruit that originated in North America and has also been successfully introduced into Europe (Fira *et al.* 2008). The dark-colored fruits contain a significant amount of anthocyanins,

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which are believed to have chemopreventive effects, and flavonoids, which act as antioxidants (Castrejón *et al.* 2008; Aqil *et al.* 2016). *Fragaria* × *ananassa* (strawberry) is a well-known and widely appreciated berry, mainly for its bright red color, juicy texture, and characteristic aroma (Ashrafuz-zaman *et al.* 2013). It is best known for its remarkably high vitamin C and folate content and is one of the most widely consumed berries, either fresh or processed into juices, jams, and syrups (Giamperi *et al.* 2014).

Cryopreservation is an ideal method for long-term storage of germplasm since preservation of clonal genotypes of most fruit crops requires time-consuming vegetative propagation. Plant material can be stored without modification for a theoretically unlimited period of time, is protected from contamination, and requires very little maintenance (Engelmann 2004; Reed 2008). Cryopreservation serves various purposes (Panis and Thinh 2001). It facilitates hybridization between plants with differing flowering schedules and allows for hybridization between geographically separated plant populations. Furthermore, cryopreservation aids in reducing the transmission of diseases through pollination vectors. Importantly, it minimizes the risk of somaclonal variation, typically encountered in tissue culture, and gametoclonal variation, which occurs in field collections. Consequently, this method enables the establishment of cultures characterized by both vegetative propagation and genetic uniformity among clones (Roque-Borda *et al.* 2021).

Large-scale use of cryogenic storage in small fruit research is hampered by genotype-specific responses to cryoprotective treatments. Thus, there is a need to develop protocols for lesser-known species and optimize existing protocols as well as to use promising techniques, such as V- and D cryo-plate (Niino *et al.* 2013; Yamamoto *et al.* 2011). These are less laborious and result in enhanced regrowth potential compared to the conventional cryogenic methods (Matsumoto 2017). As a result, high regrowth percentages are anticipated following cryopreservation.

To date, vitrification-based cryopreservation methods have been used in most cases for cryopreservation of highbush blueberry and strawberry. For *V. corymbosum*, protocols for encapsulation-dehydration (Uchendu and Reed 2009), vitrification (Kami *et al.* 2009), and droplet-vitrification (Wang *et al.* 2017) have been successfully applied. No V cryo-plate developed protocol has been described for this species so far, although some researchers have already applied this method to other *Vaccinium* spp., such as *V. ashei* and *V. virgatum* (Matsumoto *et al.* 2014; Takimoto *et al.* 2020). The D cryo-plate method was used so far in only one study focused on five *V. corymbosum* cultivars (Dhungana *et al.* 2017). For *F. ananassa*, methods for encapsulation-dehydration (Höfer and Reed 2010), vitrification (Niino *et al.* 2003), and droplet-vitrification (Pinker *et al.* 2009) have already

been developed. Cryo-plate methods are less applied for this species. Yamamoto *et al.* (2012) used the V cryo-plate method for cryopreservation of shoot tips of 15 strawberry cultivars with an average regrowth of 81%, while the D cryo-plate method was described so far in a study focused on *F. ananassa* cultivar 'Earliglow' with maximum regrowth of 40% (Gupta and Tewari 2020). In the case of *A. alnifolia* (Nutt.) M. Roem., no cryopreservation protocol has been developed yet.

This work was undertaken in response to the strikingly limited number of published studies on highbush blueberry and strawberry, and the notable absence of any published results related to saskatoon when considering the application of cryo-plate methods. The study aims to investigate the efficiency of V cryo-plate and D cryo-plate methods in successfully cryopreserving and regrowing these three small fruit cultivars. In addition, the study evaluates the multiplication index as well as the length of axial and axillary shoots regenerated from cryopreserved explants. This investigation represents a novel approach as, to the best of present authors' knowledge, the multiplication capacity of post-cryopreserved shoots has not been previously studied in these species. The findings will provide valuable insights for determining the most suitable method for each cultivar and understanding the impact of cryopreservation on the regenerative potential of treated plants.

Material and Methods

Plant Material and Establishment of Aseptic Culture Highbush blueberry cultivar 'Toro' (*V. corymbosum*), strawberry cultivar 'Clery' (*F. ananassa*), and saskatoon (*A. alnifolia* (Nutt.) M. Roem.) were included in the experiments. Aseptic cultures of highbush blueberry and saskatoon were initiated at the Institute of Plant Genetics and Biotechnology SAS, Nitra, Slovakia, while that of strawberry was established at the Fruit Research Institute, Čačak, Serbia. All cryopreservation experiments were performed in the Tissue Culture Laboratory of Fruit Research Institute Čačak.

Highbush blueberry and saskatoon sprouts bearing several axillary or apical buds were taken from mature plants, cut into smaller pieces (1 to 1.5 cm), and sterilized with 70% (v/v) ethanol for 2 min followed by immersion in 0.1% (w/v) HgCl₂ (Sigma-Aldrich, St. Louis, MO) with a few drops of Tween 20 (Sigma-Aldrich) for 5 min along with three rinses in sterile distilled water. In strawberry, runner tips (1 to 2 cm) were excised from mother plants during the growing season in the greenhouse. Disinfection was performed by immersing in 70% ethanol for 1 min and then in 1.0% (v/v) NaOCl (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 15 min followed by triple rinsing in sterile distilled water. Following sterilization, single-node

explants of highbush blueberry and saskatoon and shoot tips excised from strawberry runners were placed in sterile Petri dishes (6 cm in diameter) filled with the culture medium. *In vitro* donor cultures were established on full-strength Murashige and Skoog medium (MS, Murashige and Skoog 1962, Duchefa Biochemie B.V., Haarlem, NL) for strawberry and saskatoon and on medium with macro-salts reduced to $\frac{1}{2}$ in highbush blueberry. All media were supplemented with 30.0 g L^{-1} sucrose (Slavus, Bratislava, SK) and 8.0 g L^{-1} plant agar (Duchefa Biochemie). The pH of the media was adjusted to 5.6 (full-strength MS) or 4.8 (MS with half-strength macro-salts) before autoclaving for 20 min at 121°C and 120 kPa. All plant growth regulators (PGR) were filter-sterilized before addition to the culture medium. Growth regulators (all from Duchefa Biochemie) for shoot initiation were used as follows: 1.0 or 2.0 mg L^{-1} 6-benzylaminopurine (BAP) combined with 0.5 mg L^{-1} indole-3-butyric acid (IBA) for saskatoon (Hunková *et al.* 2021) and strawberry (Ashrafuzzaman *et al.* 2013), respectively, and 2.0 mg L^{-1} zeatin with 0.2 mg L^{-1} indole-3-acetic acid (IAA) for highbush blueberry, respectively (Hunková *et al.* 2023). After initiation, shoots from each genotype were subcultured at 4-wk intervals on medium with constant PGR composition to obtain a sufficient number of mother stock axillary shoots for cryopreservation experiments. All cultures were maintained in a growth chamber at $23 \pm 1^\circ\text{C}$ with a 16-h light/8-h dark photoperiod under cool white-fluorescent lights at a photosynthetic photon flux density $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Alfaco inženjering d.o.o., Čačak, Serbia).

Explant Isolation and Pretreatment Shoot tips (1.5 mm long) excised from 6-wk-old plantlets grown *in vitro* were used as initial explants for cryopreservation experiments. After dissection, explants were grown for 24 h in the dark at 23°C on a solid multiplication medium supplemented with 0.3 M sucrose. For highbush blueberry, MS with macro-salts reduced to $\frac{1}{2}$ (micro-salts and vitamins unchanged, Duchefa Biochemie B.V.) with 2.0 mg L^{-1} zeatin, 0.2 mg L^{-1} IAA, and pH adjusted to 4.8 was used as the basal medium. For strawberry and saskatoon, the basal medium was a full-strength MS medium with 1.0 mg L^{-1} BA, 0.1 mg L^{-1} IBA, and 0.1 mg L^{-1} gibberellic acid (GA_3) with 0.3 M sucrose and pH set to 5.6. The stated PGR combination was previously determined as the most suitable in the multiplication stage of tested genotypes (data are not presented).

Cryopreservation Aluminum cryo-plates (7 mm $\times$ 37 mm $\times$ 0.5 mm; Taiyo Nippon Sanso Corp., Tokyo, JP) that fit into 2.0-mL cryotubes with 10 or 12 oval-shaped wells [2.5 mm (L) $\times$ 1.5 mm (W) $\times$ 0.75 mm (D)] were used as explants carriers (Yamamoto *et al.* 2011) in

both V cryo-plate and D cryo-plate protocol. Excised shoot tips were plated in wells of aluminum cryo-plate filled with approximately 4.0 μL of 2% (w/w) Na-alginate (Sigma-Aldrich) solution in a calcium-free basal medium containing 0.4 M sucrose. Then, 0.1 M CaCl_2 (Carl Roth GmbH + Co. KG) solution supplemented with 0.4 M sucrose in the basal medium was poured on the aluminum plates with shoot tips and left for 30 min for polymerization. After removing the CaCl_2 solution by gently tapping onto filter paper, the cryo-plates with attached shoot tips were treated with a loading solution (LS) containing 2 M glycerol (Carlo Erba Reagents srl, Cornaredo MI, IT) and 0.8 M sucrose. Osmoprotection was performed for 30 min at room temperature.

In the protocol for V cryo-plate, explants were dehydrated at room temperature with original PVS2 solution (13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol (EG, Carlo Erba Reagents srl), and 15% dimethylsulfoxide (DMSO, Carlo Erba Reagents srl)) (Sakai *et al.* 1990); 90% PVS2 solution–PVS A3 solution (22.5% sucrose, 37.5% glycerol, 15.0% EG, and 15.0% DMSO) (Kim *et al.* 2009); and PVS3 solution (50.0% glycerol and 50.0% sucrose) (Nishizawa *et al.* 1993). Highbush blueberry shoot tips were dehydrated for 20, 40, and 50 min with each vitrification solution (VS). Based on the best regrowth percentages in this genotype, 50-min treatment was chosen as the most efficient for all three VS types and was applied for cryopreservation of saskatoon and strawberry. The protocol for the D cryo-plate method consisted of the same steps as the V cryo-plate procedure except for the dehydration step, which was performed by desiccation in closed 100-mL glass containers over 40.0 g of silica gel (Carl Roth GmbH + Co. KG) at 23°C for 2, 2.5, and 3 h. After dehydration or desiccation, cryo-plates with shoot tips were transferred to uncapped 2.0-mL cryotubes (Carl Roth GmbH + Co. KG) held on a cryo-cane and directly immersed in liquid nitrogen (LN) for 1 h.

For rapid warming and unloading, cryo-plates with shoot tips were transferred directly from LN to 1 M sucrose solution for 15 min at 25°C . Then, shoot tips were placed on solidified regeneration medium (corresponds to those determined to be most suitable in the multiplication stage). Shoots were cultivated in the dark for 7 d and then under standard conditions in the growth room ($23 \pm 1^\circ\text{C}$; 16-h light/8-h dark photoperiod; $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light intensity).

Assessment of Growth Recovery Regrowth was specifically defined as the regeneration of viable shoots from the shoot tips within the first 8 wk of cultivation. Regrowth percentages were expressed relative to the total number of shoot tips treated. The mean number of regenerated shoots per explant was determined by dividing the total number of obtained shoots by the number of explants that showed regeneration in each treatment.

Three independent replicates of approximately 12 shoot tips per each replicate were tested in each experimental treatment for each individual genotype. Therefore, 972 explants were isolated for highbush blueberry (27 treatments $\times$ 3 replicates $\times$ 12 explants) while 540 explants were isolated per genotype for strawberry and saskatoon (15 treatments $\times$ 3 replicates $\times$ 12 explants per each genotype). If the shoot tips were treated with LN for at least 1 h, they were considered as cryopreservation treatments and referred to as “+LN.” When treatments did not include the cooling explants in LN, they were considered controls and labeled as “–LN.” Therefore, the experiments included the following controls: dissection control, pregrowth control, loading control, dehydration, and desiccation control.

Monitoring of Shoot Multiplication Parameters After regeneration, shoots derived from different treatments were transplanted separately onto a multiplication medium (previously defined for each genotype as the most suitable for multiplication), labeled according to their origin, and subcultured for two consecutive cycles (each lasting 28 d) on a medium with constant composition. At the end of the second subculture, multiplication parameters including multiplication index and length of axial and axillary shoots were determined. Multiplication index was defined as the number of newly formed shoots (longer than 5 mm) per initial shoot planted at the beginning of the second subculture. All parameters were measured on at least 30 randomly selected plantlets of different origins in each genotype (three replicates with at least of 10 plantlets per replicate).

Statistical Analysis For comparison and determination of significant differences among means ($P \leq 0.05$), one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed using Statistica 12.0 package for Windows (StatSoft, Inc., Tulsa, OK). Data presented as percentages were subjected to an arcsine transformation before ANOVA to stabilize the variance of the data.

Results

Cryopreservation of Highbush Blueberry Concerning regeneration, the highest percentage of regrowth was observed in the controls for dissection, pretreatments, and loading. However, the other treatments (dehydration, desiccation, and cryopreservation) generally resulted in a decrease of shoot regeneration ability for both applied methods (Table 1). Comparison of dehydration controls with the corresponding cryopreserved explants showed that LN exposure significantly affected the regenerative capacity of the explants. The percentages of regeneration in PVS2 dehydration controls were above 90%, whereas in cryopreserved explants,

Table 1. Regrowth of dissection, pregrowth, loading, dehydration, and desiccation (–LN) controls and explants of highbush blueberry *Vaccinium corymbosum* (Ciocărlan 1990) ‘Toro’ cryopreserved (+LN) using V cryo-plate and D cryo-plate methods

Treatment and duration	Regrowth (%)		Number of regenerants per explant	
	–LN	+LN	–LN	+LN
Dissection control	100.0a	n/p	1.9a–g	/
Pregrowth control	100.0a	n/p	1.8b–h	/
Loading control	100.0a	n/p	2.2abc	/
PVS2 20 min	100.0a	8.3g	1.4f–j	1.5e–j
PVS A3 20 min	91.7b	14.2g	1.7b–i	1.3h–j
PVS3 20 min	100.0a	0.0h	2.3ab	/
PVS2 40 min	91.7b	33.5f	2.4a	1.5e–j
PVSA3 40 min	91.7b	37.7f	1.6c–i	1.0j
PVS3 40 min	66.7de	68.4de	1.4f–j	1.2ij
PVS2 50 min	100.0a	61.7e	2.1a–d	1.8b–h
PVS A3 50 min	100.0a	80.9bc	1.4f–j	1.8b–h
PVS3 50 min	100.0a	76.4cd	1.7b–i	2.1a–d
2-h desiccation	83.3c	79.8cd	2.0a–e	1.5e–j
2.5-h desiccation	88.9bc	89.4bc	2.0a–e	1.3h–j
3-h desiccation	90.0bc	87.5bc	2.0a–e	1.6c–i
	$P \leq 0.05$		$P \leq 0.05$	

Mean values of each parameter (regrowth and number of regenerants per explant), which were arranged in column pairs and labeled with the same small letter, are not significantly different according to Duncan's multiple range test; regrowth—regeneration of viable shoots from the shoot tips during 8 wk of cultivation; LN—liquid nitrogen; PVS2—plant vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose; n/p—experiment was not performed; /—experiments were not performed or no regrowth was achieved after treatment, and therefore, corresponding data are missing

these percentages were significantly lower and varied across a broad range, depending on the treatment duration. The highest percentage of regeneration for cryopreserved explants was observed in the 50-min PVS2 treatment (Fig. 1a). Dehydration with PVS A3 solution also resulted in high regeneration percentages (above 90%) for control samples and significantly lower values for corresponding cryopreserved explants. The best regrowth of cryopreserved explants (around 80%) was achieved also with the 50-min treatment (Fig. 1b). PVS3 dehydration exhibited varying regenerative trends for controls, with the highest regrowth rates observed for these samples. On the other hand, cryopreserved explants displayed a range of regrowth percentages, with the 50-min PVS3 treatment yielding the most favorable outcome—above 75% (Fig. 1c). In contrast, the D cryo-plate method protocol resulted in a less significant reduction in regenerative capacity after cryopreservation,

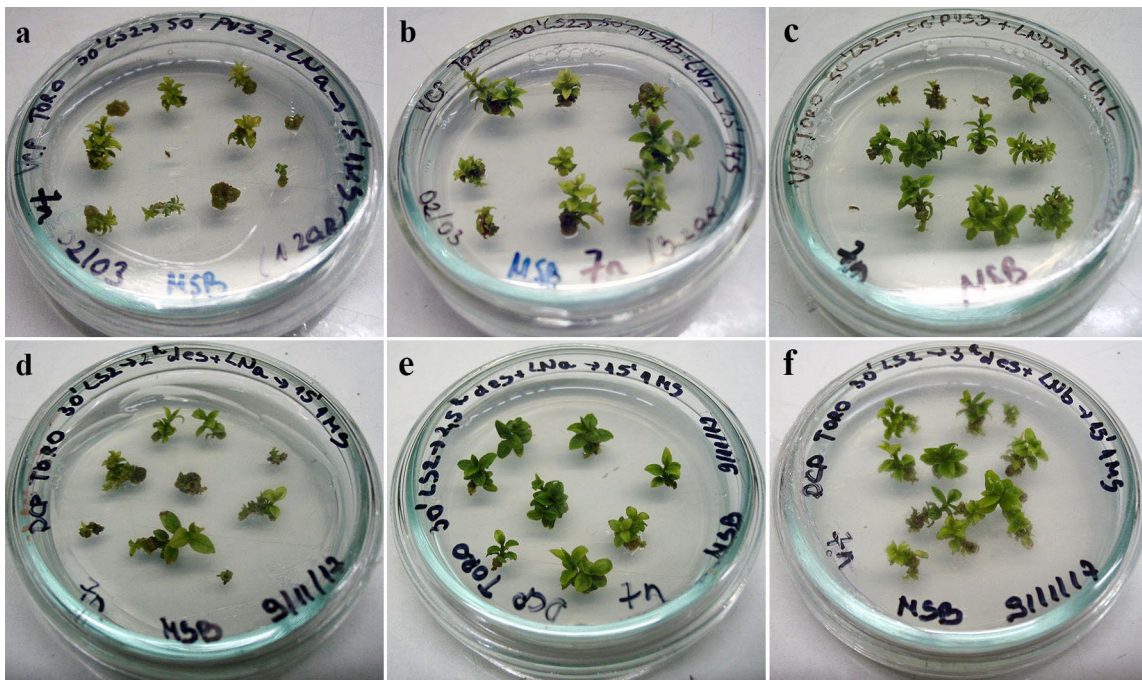


Figure 1. Regrowth of highbush blueberry *Vaccinium corymbosum* (Ciocârlan 1990) ‘Toro’ shoot tips cryopreserved by V cryo-plate method—explants dehydrated for 50 min at room temperature using PVS2 (a), PVS A3 (b), and PVS3 (c); and D cryo-plate method—explants desiccated for 2 h (d), 2.5 h (e), and 3 h (f). The diameter of Petri dish was 5.5 cm. PVS2—plant vitrification solution comprising

13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose.

with similar regeneration percentages observed for desiccation controls and cryopreserved explants (Fig. 1d–f). Very high regrowth percentages (approximately 80% or higher) were consistently attained in cryopreserved explants, irrespective of the desiccation duration (Table 1).

The average number of regenerated shoots per explant ranged from 1 to 2.4 in the V cryo-plate protocol and from 1.3 to 2 in the D cryo-plate protocol with no significant differences observed between cryopreserved explants and the corresponding dehydration and desiccation controls (Table 1). Shoots regenerated from explants after both V and D cryo-plate methods were small but green and healthy without any chlorosis or necrosis (Fig. 1).

Cryopreservation of Strawberry and Saskatoon Based on the best results obtained with the V cryo-plate method for highbush blueberry cryopreservation, the 50-min treatments with PVS2, PVS A3, and PVS3 were selected for cryopreservation experiments in strawberry and saskatoon (Tables 2 and 3). As for the D cryo-plate method, all three desiccation times were tested.

In strawberry, all steps following pregrowth treatment (loading, dehydration, desiccation, and cryopreservation) significantly reduced the percentage of shoot regeneration for both methods (Table 2). However, comparing the dehydration and desiccation controls with

the corresponding cryopreserved explants, it was found that exposure to LN had no significant effect on explant regrowth in most treatments. A significant decrease in regenerative capacity was observed only in PVS2 treatment (Table 2). In the D cryo-plate method protocol, regeneration in control samples consistently exceeded 60% across all treatments and exhibited a narrower range of variation compared to the corresponding cryopreserved explants, where regrowth ranged from just above 45% to slightly over 80% (Table 2). Overall, the highest regrowth of cryopreserved explants was achieved with PVS3 dehydration and after 2.5 h of desiccation. The number of regenerants per explant in the V cryo-plate protocol ranged from 3 to 5.3 for dehydration controls and 2.7 to 4 for cryopreserved explants. Lower values of this parameter were recorded in the D cryo-plate protocol, ranging from 2.3 to 2.6 for desiccation controls and 2.5 to 3.8 for cryopreserved explants. Compared with pregrowth and loading controls, these values were not statistically different (Table 2). Shoots regenerated from explants cryopreserved by the V cryo-plate method were well developed and had a large number of bright green, large leaves showing signs of hyperhydricity (Fig. 2a–c). Shoots regenerated from explants cryopreserved using the D cryo-plate method were less vigorous, with smaller leaves, but without signs of hyperhydricity (Fig. 2d–f).

Table 2. Regrowth of dissection, pregrowth, loading, and dehydration/desiccation (–LN) controls and explants of strawberry *Fragaria × ananassa* ‘Clery’ cryopreserved (+LN) using V cryo-plate and D cryo-plate methods

Treatment and duration	Regrowth (%)	Number of regenerants per explant		
		–LN	+LN	+LN
Dissection control	100.0a	n/p	2.5cd	/
Pregrowth control	100.0a	n/p	2.6cd	/
Loading control	83.3b	n/p	2.9b	/
PVS2 50 min	80.0b	50.0de	3.9b	2.7cd
PVS A3 50 min	55.6de	45.9e	3.0b	4.0b
PVS3 50 min	50.0de	62.5cd	5.3a	3.8b
2-h desiccation	63.6cd	62.5cd	2.6cd	2.5cd
2.5-h desiccation	83.3b	83.3b	2.3d	2.9b
3-h desiccation	75.0bc	46.2e	2.5cd	3.8b
	$P \leq 0.05$		$P \leq 0.05$	

Mean values of each parameter (regrowth and number of regenerants per explant), which were arranged in column pairs and labeled with the same small letter, are not significantly different according to Duncan's multiple range test; regrowth—regeneration of viable shoots from the shoot tips during 8 wk of cultivation; LN—liquid nitrogen; PVS2—plant vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose; n/p—experiment was not performed; /—experiments were not performed or no regrowth was achieved after treatment, and therefore, corresponding data are missing

In saskatoon, the highest regrowth was also observed in the dissection, pregrowth, and loading controls while the other treatments (dehydration, desiccation, and cryopreservation) reduced the percentage of shoot regeneration in most cases (Table 3). Using the V cryo-plate method, the lowest regeneration percentage in control samples was 50%, and it reached 100% in the case of PVS3 treatment, while regrowth in cryopreserved explants consistently remained at 50% or lower, with no regrowth observed in the case of PVS2 treatment (Fig. 3a–c). PVS3 dehydration resulted in the highest percentage of regeneration of cryopreserved shoot tips. The D cryo-plate method proved to be significantly less effective in cryopreserving this genotype. The regrowth percentages were low in both desiccation controls and the corresponding cryopreserved explants (Fig. 3d–f), with the highest value being achieved after 2 h of desiccation. The average number of regenerated shoots per cryopreserved explant ranged from 2.5 to 3.5 in the V cryo-plate protocol and from 1 to 1.1 in the D cryo-plate protocol (Table 3). Shoots regenerated from explants after the V cryo-plate method with PVS A3 and PVS3 were vigorous and green with no signs of hyperhydricity. However, almost no shoots were observed after D cryo-plate treatment (Fig. 3).

Table 3. Regrowth of dissection, pregrowth, loading, and dehydration/desiccation (–LN) controls and explants of saskatoon *Amelanchier alnifolia* (Nutt.) M. Roem. (Wisskirchen *et al.* 1998) cryopreserved (+LN) using V cryo-plate and D cryo-plate methods

Treatment and duration	Regrowth (%)		Number of regenerants per explant	
	–LN	+LN	–LN	+LN
Dissection control	100.0a	n/p	1.1d	/
Pregrowth control	100.0a	n/p	1.8c	/
Loading control	100.0a	n/p	1.1d	/
PVS2 50 min	66.7b	0.0 f	1.0d	/
PVS A3 50 min	50.0c	36.4cd	1.0d	2.5b
PVS3 50 min	100.0a	50.0c	1.0d	3.5a
2-h desiccation	25.0de	20.8e	1.0d	1.0d
2.5-h desiccation	16.7e	4.2f	1.0d	1.0d
3-h desiccation	25.0de	12.9e	1.0d	1.1d
	$P \leq 0.05$		$P \leq 0.05$	

Mean values of each parameter (regrowth and number of regenerants per explant), which were arranged in column pairs and labeled with the same small letter, are not significantly different according to Duncan's multiple range test; survival—regeneration of viable shoots from the shoot tips during 8 wk of cultivation; LN—liquid nitrogen; PVS2—plant vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose; n/p—experiment was not performed; /—experiments were not performed or no regrowth was achieved after treatment, and therefore, corresponding data are missing

Multiplication of Regenerated Shoots To evaluate the effect of each step in the applied cryopreservation protocols on the multiplication capacity of regenerated shoots in the second subculture after regrowth, a one-way ANOVA was used. The index of multiplication and the length of axial and lateral shoots were determined in shoots of different origins for each of the three genotypes studied (Table 4).

When compared with the dissection controls (shoot tips placed on regrowth medium directly after dissection), both pregrowth and loading treatments resulted in a slight decrease in multiplication capacity of regenerated shoots in highbush blueberry and saskatoon. Contrary results were observed in strawberry where a significantly higher value of this parameter was determined in shoots regenerated from loading controls compared to those originating from dissection controls. The 50-min dehydration of highbush blueberry and strawberry explants with PVSs mostly had no significant effect on the multiplication of regenerated shoots. However, an exception was the PVS A3 treatment, which resulted in a significant increase in the multiplication index of highbush blueberry shoots regenerated from cryopreserved explants. Moreover, this treatment resulted in a significant decrease in the parameter of strawberry shoots originating from both

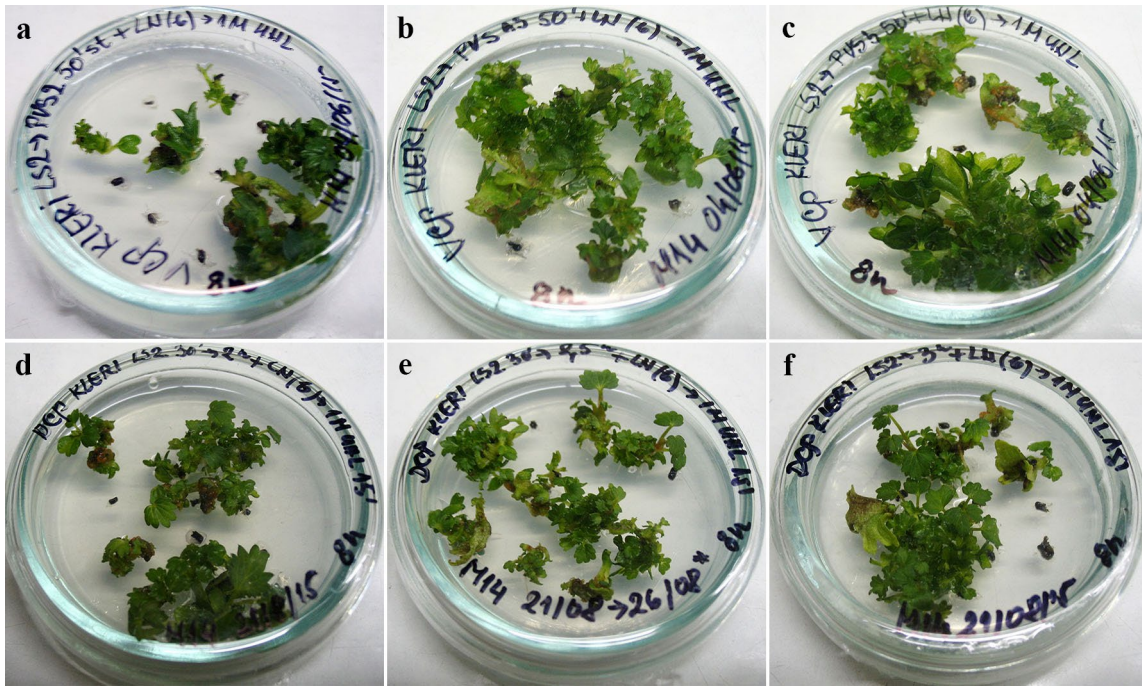


Figure 2. Regrowth of strawberry *Fragaria × ananassa* 'Clery' shoot tips cryopreserved by V cryo-plate method—explants dehydrated for 50 min at room temperature using PVS2 (a), PVS A3 (b), and PVS3 (c); and D cryo-plate method—explants desiccated for 2 h (d), 2.5 h (e), and 3 h (f). The diameter of Petri dish was 5.5 cm. PVS2—plant

vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50% glycerol and 50% sucrose.

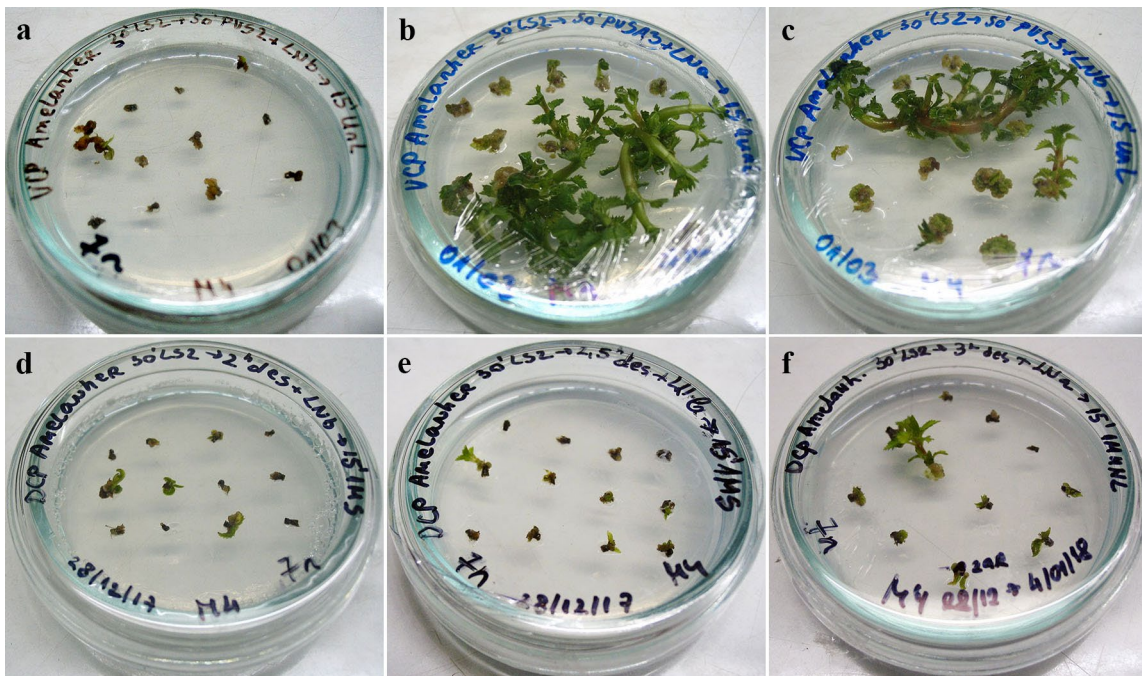


Figure 3. Regrowth of saskatoon *Amelanchier alnifolia* (Nutt.) M. Roem. (Wisskirchen *et al.* 1998) shoot tips cryopreserved by V cryo-plate method—explants dehydrated for 50 min at room temperature using PVS2 (a), PVS A3 (b), and PVS3 (c); and D cryo-plate method—explants desiccated for 2 h (d), 2.5 h (e), and 3 h (f). The diameter of Petri dish was 5.5 cm. PVS2—plant

vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose.

Table 4. Multiplication parameters of highbush blueberry, strawberry, and saskatoon *Amelanchier alnifolia* (Nutt.) M. Roem. (Wisskirchen *et al.* 1998) shoots regenerated from explants cryopreserved by V cryo-plate and D cryo-plate methods in comparison with those regenerated from control explants in the 2nd subculture after regrowth

Genotype and origin of shoots	<i>V. corymbosum</i> 'Toro'			<i>F. ananassa</i> 'Clery'			<i>A. alnifolia</i> (Nutt.) M. Roem		
	Multiplication index	Length of axial shoot (mm)	Length of lateral shoots (mm)	Multiplication index	Length of axial shoot (mm)	Length of lateral shoots (mm)	Multiplication index	Length of axial shoot (mm)	Length of lateral shoots (mm)
Dissection control	3.0bc	15.1cd	7.2ef	3.0bc	7.0fg	6.0abcd	4.2a	19.6bc	12.3b
Pregrowth control	2.3def	13.4efg	7.9cde	2.6c	6.9fg	5.4de	3.8b	18.4c	10.7c
Loading control	2.7cde	15.9bc	8.9bc	3.3a	8.7cd	5.9abcde	3.7b	18.3c	12.4b
PVS2 50 min – LN	2.7cde	12.3gh	7.2ef	2.9bc	10.1ab	6.5a	3.6bc	15.1ef	7.9 de
PVS2 50 min + LN	3.0bc	12.5fgh	7.7de	3.1ab	9.4bc	6.4a	/	/	/
PVS A3 50 min – LN	3.3b	14.5de	9.9b	2.2d	9.9ab	6.4a	4.0ab	10.6h	6.7e
PVS A3 50 min + LN	3.9a	13.9def	8.2cde	2.0d	10.4a	6.2ab	3.6bc	14.0f	8.0d
PVS3 50 min – LN	2.3ef	17.1b	11.0a	2.9bc	8.6cd	6.1abc	3.2de	13.4fg	7.8de
PVS3 50 min + LN	3.2b	18.7a	9.7b	2.8c	8.0de	6.0abcd	3.8b	12.0gh	7.2de
2-h desiccation – LN	2.0f	12.1gh	7.1ef	2.0d	7.4ef	5.6bcde	2.9e	18.1cd	12.4b
2-h desiccation + LN	2.4def	11.3hi	8.4cd	2.1d	7.0fg	5.4de	2.9e	16.3de	10.7c
2.5-h desiccation – LN	2.5de	17.3b	8.3cd	2.0d	6.4g	5.5cde	3.1de	20.7b	13.1ab
2.5-h desiccation + LN	3.3b	13.3efg	7.3de	2.0d	6.6fg	5.5cde	3.2de	20.3b	12.6b
3-h desiccation – LN	2.1f	11.3hi	7.0ef	2.0d	7.1fg	5.3e	3.3cd	20.7b	12.3b
3-h desiccation + LN	2.4def	10.2i	6.4f	2.2d	6.5fg	5.3e	3.8b	24.1a	14.0a

For each genotype, mean values of each parameter (multiplication index, length of axial shoot, and length of lateral shoots) within a single column, which are labeled with the same small letter, are not significantly different according to Duncan's multiple range test ($P \leq 0.05$). Multiplication index—the number of newly formed shoots (longer than 5 mm) per initial shoot planted at the beginning of the 2nd subculture; + LN—cryopreserved explants; – LN—non-cryopreserved explants; PVS2—plant vitrification solution comprising 13.7% sucrose, 30.0% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide; PVS A3—90% PVS2 (22.5% sucrose, 37.5% glycerol, 15.0% ethylene glycol, and 15.0% dimethylsulfoxide); PVS3—plant vitrification solution comprising 50.0% glycerol and 50.0% sucrose; /—no regrowth was achieved after treatment, and therefore, corresponding data are missing

control and cryopreserved explants. Similarly, in saskatoon, a significant decrease in the multiplication capacity of shoots regenerated from explants dehydrated with all VSs (both control and cryotreated) was observed. In terms of desiccation, this step in the D cryo-plate procedure caused a significant decrease in the multiplication capacity of both control and cryopreserved shoots in all genotypes.

Lengths of axial and lateral shoots varied significantly with the highest and lowest values observed in shoots regenerated from dehydrated, desiccated explants. In highbush blueberry, the longest axial and lateral shoots were measured in those regenerated from PVS3-dehydrated explants, while the shortest shoots were obtained from cryopreserved explants desiccated for 3 h. In strawberry, dehydration of both control and cryopreserved explants with VSs resulted in regeneration of significantly longer axial and lateral shoots compared with those originated from explants desiccated over silica gel. However, completely opposite results were observed in saskatoon where the longest axial and lateral shoots were observed in plantlets regenerated from cryopreserved explants desiccated for 3 h.

Discussion

Modern cryopreservation techniques rely on vitrification, which involves rapid cooling of plant material to a temperature below the glass transition point, at which intracellular and extracellular water transitions into an amorphous, glass-like state without the formation of damaging ice crystals (Benson 2008). Therefore, the critical step for successful recovery growth in vitrification-based methods is the dehydration process (Sakai and Engelmann 2007), which can be achieved by exposing samples to vitrification solutions or by air dehydration at temperatures above freezing. Among the various vitrification-based cryotechniques that have been successfully applied to numerous plant species (Engelmann 2014; Matsumoto and Niino 2014), droplet-vitrification (Panis *et al.* 2005) and cryopreservation using cryo-plates (Yamamoto *et al.* 2011) allow for rapid cooling and rewarming rates that can minimize the cell exposure to extreme temperatures and reduce the potential for cellular damage. As a result, they enable higher post-rewarming recovery compared to some other cryopreservation methods.

Dehydration with highly concentrated VSs is one of the most critical steps in developing any vitrification-based cryopreservation protocol. The V cryo-plate method was designed to streamline the cryopreservation process by attaching plant explants to a reusable aluminum carrier that reduces the need for excessive handling, minimizes

loss of plant material, and allows precise control over the timing of dehydration (Niino *et al.* 2019). To the present authors' knowledge, there are no previous reports on the use of the V cryo-plate method for cryopreservation of *in vitro* shoot tips of *V. corymbosum*. Matsumoto *et al.* (2014) utilized a PVS2-based V cryo-plate method for cryopreservation of dormant buds in ten *V. virgatum* cultivars. The study reported the highest regrowth of 84% with the 30-min treatment. The results in the present study demonstrated the feasibility of 50-min treatments with PVS2, PVS A3, and PVS3, which resulted in significant increase in regrowth success of cryopreserved explants with maximum over 80%. Determining the optimal duration of exposure to VSs is crucial to achieve the right balance between toxicity and sufficient dehydration of explants, thereby minimizing the risk of ice crystal formation in cryopreserved tissues (Panis *et al.* 2005). In the current study, dehydration controls for the 20- and 40-min treatments with all three VSs also exhibited a high regrowth potential, exceeding 90% for most treatments; however, corresponding cryopreserved explants had significantly lower regeneration capacity, particularly for the 20-min treatment; Uchendu and Reed (2009) employed the PVS2-based vitrification technique for the cryopreservation of shoot tips of cultivars 'Berkeley', 'O'Neal', and 'Brigitta Blue' (*V. corymbosum*). In their research, 20-min dehydration at 25°C using this VS enabled regrowth ranging from 33 to 87% with the highest regrowth observed in the 'Brigitta Blue'. In the present study, greater resistance of 'Toro' to the chemical toxicity of the PVS2 solution at 25°C was observed, and the success of viable shoot regeneration increased significantly with prolonged dehydration up to treatment duration of 50 min. It is worth noting that in the present study, the previous preconditioning of mother plants and preculture of isolated shoot tips were not applied as performed in the experiments by Uchendu and Reed (2009). In a separate study conducted by Wang *et al.* (2017) that focused on PVS2-based droplet-vitrification of various *V. corymbosum* cultivars, they achieved regrowth percentages ranging from 47 to 91%. The authors determined that a treatment duration of 40 min was optimal, which is shorter than the 50-min treatment duration determined as the most suitable in the present experiment. Although tolerance to chemical toxicity of vitrification solutions may be genotype-dependent, the increased tolerance to prolonged PVS2 treatment observed in the present experiment may be due to the possible mitigation of chemical toxicity by the calcium alginate gel used to attach shoot tips to aluminum plates (Yamamoto *et al.* 2011).

Comparing the VSs among themselves, it can be concluded that PVS A3- and PVS3-based dehydration were superior to PVS2 dehydration in highbush blueberry

‘Toro’. In addition, markedly lower proliferation capacity of shoots regenerated from control and cryopreserved explants treated with PVS2 compared with PVS A3 and PVS3 dehydrated shoot tips indicates that this genotype is more sensitive to the cytotoxicity of this VS. This agrees with previous studies of present authors focused on droplet-vitrification technique used for cryopreservation of blackberry ‘Čačanska Bestrna’ (Vujović *et al.* 2011, 2015a). Kim *et al.* (2009) demonstrated that a balanced composition of PVS A3 solution can be more beneficial for the recovery of cryopreserved samples compared to the original PVS2 solution, while PVS3 solution is recommended to use for samples sensitive to chemical toxicity and tolerant to osmotic stress. To the present authors’ knowledge, no other study has compared the effect of PVS A3 and PVS3 on highbush blueberry cryopreservation.

Considering that the 50-min treatment with all three VSs resulted in the highest regrowth of highbush blueberry ‘Toro’ (Ericaceae family), the present authors chose this treatment for further experiments with strawberry and saskatoon (both belonging to the Rosaceae family). This decision was made despite the recommendation that the type or modifications of VSs should be chosen from cryoprotective solutions that have shown high regeneration in species of the same family (Zamecnik *et al.* 2021).

Regarding the strawberry ‘Clery’, no significant difference in regrowth was observed between explants treated with the original PVS2 solution and PVS A3 solution. These results (regrowth around 50%) were notably lower than those obtained by Yamamoto *et al.* (2012), who achieved an average regrowth level of 81% in 15 strawberry cultivars with the V cryo-plate method using PVS2 solution for 50 min. The higher regrowth percentages observed in their study can be attributed to the improved physiological state of the donor plants achieved by preconditioning, which involved cold accumulation at 5°C for 3 to 4 wk. In an effort to simplify and speed up the cryopreservation procedure, the present authors attempted to omit the cold acclimation step and proceed directly to cryopreservation by using shoot tips isolated from plants grown under standard conditions. In the present research, the preculturing of strawberry shoot tips on agar-solidified medium with 0.3 M sucrose was probably not sufficient to achieve as high regrowth percentages after cryopreservation as those reported by Yamamoto *et al.* (2012). The importance of cold acclimation in achieving high regrowth levels after cryopreservation using other vitrification-based cryopreservation techniques in strawberries has already been emphasized by Reed and Hummer (1995) and Niino *et al.* (2003). However, the present results are comparable to those achieved in three strawberry cultivars (regrowth ranged between 40 and 67%) cryopreserved using the PVS2-based droplet-vitrification technique (Pinker *et al.* 2009), although the duration of VS treatment was twice

as long in the present study. PVS3-based dehydration for 50 min in strawberry ‘Clery’ resulted in significantly higher regrowth (above 60%) compared to that obtained with PVS2 and PVS A3. Similarly, Bae *et al.* (2021) reported average regrowth of 61.5% in 26 strawberry accessions cryopreserved using the PVS3-based droplet-vitrification protocol.

The lowest regeneration potential using the V cryo-plate method was observed in saskatoon where the regrowth reached less than 40% under dehydration with PVS2 and PVS A3 while no regrowth was observed after the use of PVS2. These results along with significant decrease in regrowth percentage of corresponding dehydration controls compared to dissection, pregrowth, and loading controls indicated that this genotype is highly sensitive to biochemical toxicity of PVS2 or its variants. Therefore, the present authors recommend that treatment should be shorter than 50 min or performed at 0°C to mitigate the detrimental effects of these VSs. On the other hand, this genotype showed tolerance to the osmotic toxicity of PVS3 solution, as evidenced by the maximum recovery of control explants and a regrowth of 50% in cryopreserved explants. Although there are no available results for saskatoon from other studies, other species from the Rosaceae family have been cryopreserved using various vitrification-based cryopreservation techniques. In a study conducted by Vujović *et al.* (2020), the percentage of regrowth using PVS A3-based droplet-vitrification increased significantly when dehydration was performed on ice (45 to 70%) compared with dehydration at room temperature (0 to 14.5%) for the same treatment duration (30 to 50 min). Regrowth of PVS3-treated shoot tips (40 to 60 min) showed no significant differences (45 to 50%), which is comparable to the present study’s results for saskatoon. Droplet-vitrification was also used in the studies by Pawłowska and Szewczyk-Taranek (2014) and Le Bras *et al.* (2014) for cryopreservation of shoot tips and buds taken from *in situ* plants of *Rosa* spp., achieving regrowth percentages up to 87% in both studies.

The D cryo-plate method was developed to address the challenges posed by the high sensitivity of certain plants to PVS2 treatment, replacing the VS treatment with air dehydration (Niino *et al.* 2014). In the present research, efficient regeneration was achieved by using this method in highbush blueberry (up to 89.4%) as well as in strawberry (up to 83.3%) with the highest regrowth percentage observed after 2.5 h of desiccation in both genotypes. These results are similar to those reported for blueberry ‘Tifblue’ (*V. ashei* J. M. Reade), which were approximately 90% (Dhungana *et al.* 2017). The authors of that study emphasized the optimization of blueberry cryopreservation, using preculture and loading treatment conditions very similar to experiments in the current study. The only difference was in the desiccation process, which, in their study, was carried out under the airflow of a laminar flow cabinet, as opposed to being

conducted in closed glass containers over silica gel. Uchendu and Reed (2009) utilized the encapsulation-dehydration technique for cryopreserving highbush blueberry; and after 6 h of desiccation, at least 80% of the encapsulated shoot tips successfully developed into shoots in all cultivars studied. However, comparable results were achieved in the present study with much shorter desiccation periods using the D cryo-plate method without any preconditioning of mother plants *in vitro*. With respect to strawberries, the outcomes in the current study surpassed the 40% maximum regrowth reported by Gupta and Tewari (2020) when utilizing the D cryo-plate protocol for 'Earliglow' strawberry. This suggests that the efficiency of post-rewarming regeneration is probably cultivar-specific. In the study of Höfer and Reed (2010), shoot regeneration of two strawberry cultivars cryopreserved by encapsulation-dehydration reached up to 90% after 6 h of dehydration. Compared to these findings, the present study indicates that the D cryo-plate method is highly efficient for cryopreservation of *F. ananassa* 'Clery'.

In contrast to highbush blueberry and strawberry, saskatoon again displayed much lower regrowth potential under the experimental conditions set up for D cryo-plate with the highest regrowth being around 25% for desiccation controls and 20% for cryopreserved specimens. The high sensitivity to chemical and physical dehydration suggested that the protocol for cryopreservation of this genotype should include preconditioning of mother plants for the acquisition of dehydration tolerance and thus achieving high levels of regrowth following cryopreservation. This requirement for cold-hardening as well as its optimal duration is species-specific (Niino *et al.* 2019). Moreover, the duration of sample dehydration directly affects the regrowth, as shown in *Rosa chinensis* L., where the 3- and 4-h treatments exceeded the 2-h treatment (Le Bras *et al.* 2014).

Cryopreservation exposes plant material to various stresses that can affect both cryopreserved specimens and regenerated plantlets (Reed *et al.* 2005). The development of an efficient and robust *in vitro* system enabling vigorous regrowth of explants retrieved from LN and rapid multiplication of regenerated shoots is of utmost importance in the cryopreservation process. Therefore, monitoring the multiplication capacity of shoots regenerated after cryopreservation is crucial for evaluating the success and effectiveness of cryopreservation protocols. It serves as a valuable indicator of the overall health and vigor of regenerated plantlets and their ability to undergo rapid clonal propagation. In the present research, plantlets regenerated from cryopreserved explants showed normal morphology and the ability to multiply. When comparing the shoots originating from cryopreserved explants with those regenerated from corresponding dehydration/desiccation controls, the present authors mostly did not observe significant differences in analyzed multiplication parameters. Interestingly, in some treatments, the cryopreserved shoots

had significantly higher multiplication index and length than the corresponding control shoots (originated from explants that were desiccated or dehydrated but not cryopreserved). Moreover, in the case of highbush blueberry and strawberry, the multiplication capacity of cryopreserved shoots derived from the V cryo-plate protocol even surpassed that of plantlets regenerated from dissection controls (explants that did not undergo any treatment following dissection). Similar findings were reported for *Prunus* sp. shoots cryopreserved using V and D cryo-plate methods (Vujović *et al.* 2015b). However, in apple shoots regenerated from cryopreserved explants using droplet-vitrification, dehydration with VS resulted in a significant reduction in multiplication capacity, especially in terms of multiplication index. This reduction was observed in comparison to shoots derived from dissection and pregrowth controls (Vujović *et al.* 2020). Conversely, cryopreservation did not have an impact on the multiplication phase of olive cultures derived from somatic embryos (Bradaï and Sánchez-Romero 2021). However, there was a notable interaction between genotype and cryopreservation concerning shoot length during the multiplication process. This phenomenon, observed in plantlets recovered from cryopreserved explants, was probably stress-induced and species-specific. Leunufna and Keller (2021) also found no significant differences in multiplication capacity among untreated, PVS2-treated, and cryopreservation-derived *in vitro* plantlets in two of the three evaluated yam (*Dioscorea* spp.) species. However, for the third species, the untreated *in vitro* plantlets exhibited a significantly lower multiplication rate compared to dehydration controls and shoots originating from cryotreated explants. According to those authors, this difference could be attributed to physiological effects that are expected to recover in subsequent subculture cycles.

Conclusions

This study is the first to present the successful application of the V cryo-plate method in highbush blueberry, as well as the utilization of both V and D cryo-plate methods in saskatoon. Furthermore, multiplication of shoots regenerated from cryopreserved explants was successful in all examined cultivars with regenerative abilities exceeding even those of control shoots originating from non-cryopreserved explants. The preserved capacity for multiplication of cryopreserved shoots confirms the effectiveness of the employed cryopreservation methods. Therefore, both V and D cryo-plate can be recommended for highbush blueberry and strawberry, but only V cryo-plate should be used for saskatoon. Further research should focus on the evaluation of the rooting ability and acclimatization success of shoots originating from cryopreserved specimens. This will enable us to comprehensively assess the success of the cryopreservation process,

ensuring that the preserved plant material retains its capacity to generate roots and establish itself in a new environment.

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Declarations

Conflict of interest The authors declare no competing interests.

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