In Vitro Cell.Dev.Biol.—Plant (2017) 53:394–401
DOI 10.1007/s11627-017-9860-3

CRYOPRESERVATION

Cryopreservation of red raspberry cultivars from the VIR *in vitro* collection using a modified droplet vitrification method

Y. V. Ukhatova[1] · S. E. Dunaeva[1] · O. Y. Antonova[1] · O. V. Apalikova[1] · K. S. Pozdniakova[1] · L. Y. Novikova[1] · L. E. Shuvalova[1] · T. A. Gavrilenko[1,2]

Received: 1 February 2017 / Accepted: 20 September 2017 / Published online: 6 October 2017 / Editor: Barbara Reed

Abstract The goal of the present study was to analyze the post-cryogenic recovery of 12 red raspberry cultivars from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources *in vitro* collection. The 1.1–1.8 mm shoot tips of microplants were subjected to cryopreservation using the modified droplet vitrification method. The current modifications to the droplet vitrification protocol included the elimination of the initial pretreatment stage of the microplants, and the use of modified media at the stages of initial micropropagation, explant isolation, and post-cryogenic regeneration. The optimized method reduced the duration of some cryopreservation stages compared to the initial protocol, and reduced the total procedure from 14 to 11 wk. This modified cryopreservation method also demonstrated a relatively high level of post-cryogenic regeneration. Depending on the genotype, the shoot recovery of explants after rewarming varied from 24.2–89.3% and averaged 58.8 ± 5.3%. There was a statistically significant influence of the genotype on the shoot recovery after rewarming. No differences in inter simple sequence repeats and in start codon targeted marker spectra were found between post-cryopreservation microplants and donor *in vitro* plants from two red raspberry cultivars.

Keywords Red raspberry cultivars · Cryopreservation · Droplet vitrification · *In vitro* collection · DNA markers

✉ T. A. Gavrilenko
tatjana9972@yandex.ru

[1] Department of Biotechnology, Federal Research Center the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), 42-44, Bolshaya Morskaya Street, St. Petersburg, Russia 190000

[2] St. Petersburg State University, 7/9, Universitetskaya Embassy, St. Petersburg, Russia 199034

Introduction

To ensure safe conservation of the vegetatively propagated crops in genebanks, it is recommended to duplicate this material in the field, *in vitro* and/or with cryo-collections (Reed *et al.* 2004). According to published reports, *Rubus* clonal accessions are mostly conserved in field collections (EU GEN RES 2017). Field and *in vitro* collections are the sources used to establish cryobanks. The cryo-collections of *Rubus* germplasm are not numerous and contain a comparatively small number of accessions cryopreserved using several methods. Table 1 summarizes the data on the numbers of red raspberry and blackberry accessions preserved employing different cryopreservation methods in the established cryogenic storage worldwide, and in the research centers in different countries where raspberry cryopreservation has just started.

The largest *Rubus* germplasm cryo-collection is maintained at the National Laboratory for Genetic Resources Preservation (NLGRP) in Ft Collins, CO (USA). It includes over 209 *Rubus* accessions (from 39 species) cryopreserved between 1996 and 2013, using the methods of slow cooling (National Council for Geocosmic Research (NCGR) 1996–2000), vitrification (NCGR 2000–2005), and droplet vitrification (NLGRP 2005–2013) (Maria Jenderek personal communication). Currently, the method of droplet vitrification (DV) developed by Panis *et al.* (2005) has broad applications for cryopreservation of a broad range of plant species (Panis *et al.* 2016). This DV method is relatively quick and simple and ensures good regeneration results after rewarming.

In addition to the studies aimed at developing new and modifying existing methods to raise efficiency of cryopreservation, one of the important research directions is the study of genetic stability of the regenerants obtained after rewarming (Castillo *et al.* 2010; Kaczmarczyk *et al.* 2011; Wang *et al.* 2014, 2017). Various DNA markers are widely used to

Table 1. *Rubus* shoot tips collections in cryopreservation worldwide

Country, organization	Number of accessions	Crop type	Cryopreservation method	Regeneration rate after rewarming, %, min-max, average	Reference
Established cryocollections					
NLGRP in Ft Collins	Over 209	*Rubus* accessions (from 39 species)	SF, Vit, DV	n/d	Maria Jenderek (personal communication)
USA/USDA ARS NCGR	173	*Rubus* accessions	SF, ED	n/d	Reed and DeNoma 2012
	25	*Rubus* species, hybrids, cultivars	ED	60.0–100.0	Gupta and Reed 2006
	4		Vit	45.0–78.0	Gupta and Reed 2006
Kazakhstan/IPBB	31	*Rubus* accessions	ED, Vit	n/d	Kovalchuk *et al.* 2014
	11	8 red raspberry cultivars, 3 raspberry selections	V	20.0–81.0	Kovalchuk *et al.* 2010
Finland/MTT	32	Raspberry accessions	DV	n/d	Nukari *et al.* 2009, 2011
Russia/VIR	13	Red raspberry cultivars	DV	24.2–89.3	Present paper
	3	Blackberry cultivar	DV	20.0–67.5	Present paper
Cryopreservation experiments					
Finland / MTT	7	4 red raspberry cultivars, 3 hybrids	EVit	50.0–85.0	Wang *et al.* 2005
India / NBPGR	5	*Rubus* hybrids	ED	13.0–80.0	Anonymous 2015
Russia/ IPPRAS	2	Red raspberry cultivars	SF	25.0–77.0	Vysotskaya and Popov 2005
Finland / MTT	1	1 raspberry clone	ED	50	Wang *et al.* 2005
Serbia / FRI	1	Red raspberry cultivar	DV	18	Condello *et al.* 2011

ED encapsulation-dehydration, *Vit* vitrification, *EVit* encapsulation-vitrification, *DV* droplet-vitrification, *SF* slow freezing, *n/d* data not shown

IPBB Institute of Plant Biology and Biotechnology at National Center of Biotechnology, Almaty, Republic of Kazakhstan; *MTT* MTT Agrifood Research Finland, Laukaa, Finland; *USDA-ARS, NCGR* United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, Oregon, USA; *NLGRP in Ft Collins CO.* National Laboratory for Genetic Resources Preservation, Ft Collins, USA; *NBPGR* National Bureau of Plant Genetic Resources, New Delhi, India; *VIR* Federal Research Center the N. I. Vavilov All-Russian Institute of Plant Genetic Resources, St.- Petersburg, Russia; *IPPRAS* Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia; *FRI* Fruit Research Institute Čačak, Serbia

monitor genetic stability and these studies have been done on *Rubus* cryo-regenerants (Castillo *et al.* 2010). In general, no differences in the recovered and control plants were found.

This present paper presents results of cryopreservation research performed at the N.I. Vavilov Institute of Plant Genetic Resources (VIR) on the *Rubus in vitro* collection held at VIR, which consists of 177 accessions. This collection contains 87 released red raspberry cultivars, from which 27 originated abroad and 60 were bred in Russia between 1920 and 2000 by various research organizations located in different ecogeographic regions of the Russian Federation. Sixty-five of the 87 accessions of red raspberry cultivars were transferred to *in vitro* cultures from individual plants of the field-grown accessions that were previously genotyped by simple sequence repeats (SSR markers) (Lamoureux *et al.* 2011), and 48 had known biochemical composition of the berries (Lefèvre *et al.* 2011). The combined chemo-thermotherapy was successfully applied for eradication of *Raspberry Bushy Dwarf Virus* (RBDV) in the *in vitro* plants (Antonova *et al.* 2015). Accessions from the *in vitro* collection of red raspberry cultivars are being indexed for the presence of bacterial contaminants once a year according to the method of Reed *et al.* 2004. Pathogen-free samples from the *in vitro* collection were used as a basis for cryopreservation.

The goal of the study was to analyze the post-cryogenic recovery of red raspberry cultivars using modifications of the droplet vitrification method. The results obtained from cryo-regeneration studies were compared with the published data on red raspberry cultivar cryopreserved by the droplet vitrification method (Nukari *et al.* 2011; Condello *et al.* 2011). In addition, the first results of genetic uniformity study of the recovered plants obtained after rewarming performed using inter-simple sequence repeat (ISSR) and start codon targeted (SCoT) markers are presented.

Materials and methods

Plant materials Red raspberry cultivars of different origins from the N.I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR) *in vitro* collection were used for cryopreservation experiments (Table 2). These cultivars were transferred into *in vitro* culture in 2010. All microplants of each cultivar were represented by the same clone because they originated from an individual field-grown plant (Lamoureux *et al.* 2011).

Table 2. List of red raspberry (*Rubus. idaeus* L.) cultivars used in cryopreservation in present study with the information on their parentages and origins

No	Cultivar name	k-VIR	Parentage	Place of origin
1	Balsam	35,447	'Newburg' × 'Rubin Bulgarski'	Bryansk, Russia,1993
2	Barnaulskaya	31,185	'Viking' × 'Usanka'	Barnaul, Russia,1961
3	Belaya Spirina	8210	Unknown	Nikolsk, Russia, before 1938
4	Kokinskaya	35,921	'Solge' × ('Novost Kuzmina' + 'Kostinbrodskaya')	Bryansk, Russia,1980
5	Meteor	35,926	'Kostinbrodskaya' × 'Novost Kuzmina'	Bryansk, Russia,1993
6	Novokitayevskaya	29,862	'Kitayevskaya' × 'Novost Kuzmina'	Kiev,Ukraine,1974
7	Progress	8293	'Marlboro' × 'Texac'	Kozlov, Russia, before 1920
8	Samarskaya Plotnaya	40,730	'Novost Kuzmina' × 'Kaliningradskaya' ('Preussen')	Samara, Russia,1986
9	Sputnitsa	35,476	'Rubin Bulgarski'y × 'Ottawa'	Bryansk, Russia,1983
10	Shartashskaya	8337	Selection from wild red raspberry	Ekaterinburg, Russia, before 1925
11	Skromnitsa	35,478	'Rubin Bulgarskiy' × 'Ottawa'	Bryansk, Russia,1983
12	Sokolenok	40,483	'Malling Jewel' open pollinated	Barnaul, Russia,1991

Data about 'Parentage' and 'Place of origin' were taken from Bologovskaja RP (1949) and from the 'Catalog of raspberry cultivars', 2017

Establishment of *in vitro* shoot cultures and tissue culture conditions Chemical reagents were obtained from Sigma-Aldrich®, St. Louis, MO, with catalog numbers indicated. For propagation, microplant cuttings were placed on agar-solidified MS (Murashige and Skoog 1962) nutrient medium (#M5519) with 2.2 μM 6-Benzylaminopurine (BA, #B3408), 0.2 μM Indole-3-butyric acid (IBA, #I5386), and 3% (*w/v*) sucrose (#S7903) (Wang *et al.* 2005). The initial medium included 7 g L^{-1} agar (#A1296) with the pH adjusted to 5.8 using 0.1 N KOH before autoclaving at 121 °C for 20 min. Growth room conditions were 16-h photoperiod (25 μM m^{-2} s^{-1}) with cool-white tubular fluorescent lamps (OSRAM T8L36W/640G13 1200 mm, OSRAM, Munich, Germany) at 23 °C (standard growth room conditions). Cultures were subcultured at 4-wk. intervals.

Cryopreservation and regrowth All reagents for cryopreservation and regrowth were obtained from Sigma-Aldrich®, St. Louis, MO, with catalog numbers indicated. The droplet vitrification method described by Panis *et al.* (2005) with modifications (Table 3) was used for cryopreservation of 12 red raspberry cultivars. Isolated shoot tips 1.1–1.8-mm long were put into liquid MS medium without growth regulators during isolation and were transferred into a filter sterilized loading solutions (LS) containing MS basal liquid medium with 2 M glycerol and 0.4 M sucrose (Matsumoto *et al.* 1994), for 20 min at 23–25 °C. The shoot tips were further incubated in filter sterilized Plant Vitrification Solution 2 (PVS2), consisting of MS basal liquid medium with 3.26 M glycerol, 2.42 M ethylene glycol (EG), 1.9 M dimethyl sulfoxide (DMSO), and 0.4 M sucrose (Sakai *et al.* 1990), for 30 min on ice. Five minutes before the end of the PVS2 treatment, the explants were put into individual droplets (3 μL) of PVS2 on sterile aluminum foil strips, and quickly plunged into cryovials filled with liquid nitrogen (LN). The cryovials were then plunged into LN. After 1 h in LN on aluminum foil, the explants were rewarmed in filter sterilized rewarming solution (RS), pH 5.8, consisting of MS basal liquid medium with 1.2 M sucrose, (Sakai 1997) for 15 min at 23–25 °C. The rewarmed explants were put on the solidified basic MS recovering medium, pH 5.8, containing 1.4 μM trans-zeatin-riboside (Sigma-Aldrich®, #Z0375), 2.9 μM 3-indoleacetic acid (IAA, #I2886), and 0.6 μM gibberellic acid (GA, #G7645) which were dissolved in distilled water and sterilized through a 0.22-μm Millipore filter (Guangzhou Jet Bio-filtration Co, Guangzhou, China) and added to autoclaved MS medium and 30 g L^{-1} sucrose (Towill 1983). The recovering medium included 7 g L^{-1} agar (#A1296) with the pH adjusted to 5.8 using 0.1 N KOH before autoclaving. The recovering medium was dispensed into sterile plastic Petri dishes (60 mm × 10 mm) after autoclaving at 121 °C, 152 kPa for 20 min. Ten explants were cultured per Petri dish and cultivated for regeneration in standard growth room conditions.

Each step of cryopreservation was performed with three replications containing 20 experimental explants per step. In total, three replications of 60 explants per accession were cryopreserved to assess the regeneration rate. Simultaneously, 10 control explants per replication were treated with all the solutions (LS, PVS2, and RS), but not immersed in liquid nitrogen. In total, there were three replications of 30 control explants per accession.

Regeneration (percent of explants which restarted growth and formed shoots) was determined on the 3rd and the 6th wk after rewarming. Data are presented as a percentage of the total number of cryopreserved explants.

Each accession for long-term storage in a cryobank (in liquid nitrogen) was processed with 90 explants, and

Table 3. Comparison of droplet-vitrification (DV) procedure applied for red raspberry cryopreservation compared to original DV method of Panis *et al.* (2005) developed for banana meristem

Droplet-vitrification procedure, place of use, references ➡	DV, INIBAP, Belgium (original protocol) (Panis *et al.* 2005)	DV, MTT ARF, Finland (Nukari and Uosukainen 2007; *et al.* 2011)	DV, FRI, Serbia (Condello *et al.* 2011)	DV, VIR, Russia—present paper
Stage of procedure				
1.Micropropagation medium: conditions, duration.	MS with 2,25 mg L^{-1} 6-Benzylaminopurine (BA), 0,17 mg L^{-1} 3-indoleacetic acid (IAA), 10 mg L^{-1} ascorbic acid, 30 g L^{-1} sucrose, at 25 ± 2 °C under continuous illumination. Shoots of 3–5 cm separated and transferred to a rooting medium (MS with 0.5 g L^{-1} activated charcoal, 10 mg L^{-1} ascorbic acid, 3 g L^{-1} Gelrite™, and 30 g L^{-1} sucrose (pH 5.8) for 4 wk	No data	MS with 0.3 mg L^{-1} BA, 30 g L^{-1} sucrose, at 25 °C, 16-h photoperiod 4–5 wk	MS with MS with 0.5 mg L^{-1} BA, 0.05 mg L^{-1} Indole-3-butyric acid (IBA), 30 g L^{-1} sucrose, at 22 ± 2 °C, 16-h photoperiod, 5 wk
2. Donor microplant preculture				
2a. Cultivation on solidified medium with improved sucrose content	Rooted plants transferred to hormone free MS medium supplemented with 60 g L^{-1} sucrose for 1 mo	Plants transferred to fresh medium supplemented with 0.25–0.75M sucrose	–	–
2b.Cold preculture	–	+	–	–
3. Preculture of the explants: media, duration				
3a. Explant isolation:	Explants transferred into LS up to 5–7 h (during the time of isolation of all explants)	Explants transferred on solid MS with AC for 3 d.	LS directly after isolation of the explants	Explants transferred into liquid hormones-free MS for about 1 h.
3b. Osmoprotection		Explants transferred into LS for 30 min at RT with light	LS 20 mins at RT in the dark	Explants transferred into LS for 20 min at RT with light
3c. Dehydration	PVS2 30–50 min on ice	PVS2 45–60 min at RT	PVS2 30 min on ice	PVS2 30 min on ice
4. Transferring of the explants	In the droplets of PVS2 on aluminum foils strips			
4. Freezing (immersion into LN)	The foil strip immediately transferred to a 2-mL cryotube filled with LN	The foil strip immediately transferred to a 2-mL cryotube filled with sterile air, then—into LN	The foil strip immediately transferred to a 2-mL cryotube filled with LN	The foil strip immediately transferred to a 2-mL cryotube filled with LN
5. Rewarming	Fast rewarming into liquid RS 15 min at RT			
6. Post-cryogenic regeneration: conditions				
First week in the dark	+	No data	+	–
Recovery medium	MS with 10 mg L^{-1} ascorbic acid, 3 g L^{-1} Gelrite™, 0.3 M sucrose (on filter paper) for 2 d, then—directly on micropropagation medium	No data	First day—on semi-solid hormone-free MS with 0.3M sucrose (agar 4.5 g L^{-1}), then—on solid MS (agar 5.5 g L^{-1}) with 0.5 mg L^{-1} BA	Solid MS (agar 7 g L^{-1}) with 0.5 mg L^{-1} trans-zeatin-rhibozide, 0.2 mg L^{-1} gibberellic acid (GA_3), 0.5 mg L^{-1} IAA, and 30 g L^{-1} sucrose
The timing of the evaluation frequency of explant recovery after thawing	On the 4th–6th wk. after thawing	No data	Only once - on the 3rd wk. after thawing	On the 3rd and on the 6th wk after thawing
Total duration	14 wk	No data	7–8 wk	11 wk

PVS2 = (*v*/*v*) 30% (*v*/*v*) glycerol, 15% (*v*/*v*) ethylene glycol and 15% (*v*/*v*) DMSO in liquid MS medium with 0.4 M sucrose, RS = 1.2 M sucrose in liquid MS medium.

"+" this stage of DV procedure is present. "−" this stage of DV procedure is absent

RT room temperature, *AC* activated charcoal, *LN* liquid nitrogen, *LS* loading solution—2 M glycerol and 0.4 M sucrose in liquid MS medium, *no data* no data available

Table 4. Primers used in genetic stability assessment of red raspberries plants regrown after cryopreservation

Type of molecular marker	Primer name	Sequence (5′–3′)	Tm	Reference
ISSR	$(AG)_8C$	$(AG)_8C$	50°C	Graham *et al.* 1994
ISSR	$(GT)_8C$	$(GT)_8C$	50°C	Graham *et al.* 1994
SCoT	SCoT4	CAACAATGGCTACCACCT	50°C	Collard and Mackill 2009
SCoT	SCoT12	ACGACATGGCGACCAACG	50°C	Collard and Mackill 2009

distributed in 9 cryogenic vials, with 10 explants in each vial. This made it possible to periodically remove individual cryogenic vials to monitor the ability of the cryopreserved shoot tips to recover.

DNA extraction All chemicals for DNA extraction and separation of amplified DNA fragments were obtained from Carl Roth® (Karsruhe, Germany, https://www.carlroth.com). Assessment of trueness to type of post-cryopreservation recovered plants to the initial *in vitro* plants was performed for two cultivars: Barnaulskaya and Samarskaya Plotnaya. All microplants of each cultivar were represented by the same clone. DNA was extracted from the donor *in vitro* plants, and from ten recovered cryo-regenerants of each cultivar. DNA was extracted by a modified cetyl trimethylammonium bromide (CTAB) extraction method as described by Gavrilenko *et al.* (2013).

PCR analysis All chemicals for PCR analysis were obtained from Dialat (Moscow, Russia http://dialat.ru/). Two ISSR and two SCoT primers were selected from Graham *et al.* (1994), Collard and Mackill (2009), respectively (Table 4). Primers were synthesized by Evrogen, Moscow (http://evrogen.ru/). PCR was performed in 20-μL reaction mixtures containing 40 ng of raspberry DNA, × 1 reaction buffer (Dialat, Moscow, Russia http://dialat.ru), 2.5 mM $MgCl_2$, 300 μM of each of deoxynucleotides, 0.8 μm of primer and, 1 unit of Taq-polymerase (Dialat). The PCR procedure included the following steps: 94°C for 4 min, followed by 37 cycles [94°C - 45 s, for 1.5 min., 72°C for 2 min] and finally 72°C for 5 min.

Separation of amplified DNA fragments The products from the polymerase chain reaction were separated on horizontal 2.5% (*w/v*) agarose gels in TBE buffer (Tris 0.089 M; H_3BO_3 0.089 M; EDTA 0.002 M; pH 8.2) at about 5 V cm^{-1} of gel length. After electrophoresis, the gels were stained with ethidium bromide. The DNA fragments were visualized by transmitted UV light.

Statistical analysis The statistical analysis of the results was conducted using conventional statistical methods (Student's *t* test). Statistical dispersion analysis was also carried out using the Program StatSoft Statistica™ 6.0 (Palo Alto, CA). Analysis of variance (ANOVA) was performed by Tukey's test. Significance was determined at $p \leq 0.05$.

Results and discussion

Cryopreservation of red raspberry cultivars Control explants of all 12 cultivars had better regrowth results than post-cryogenic explants (Table 5).

Out of the 12 cryopreserved red raspberry cultivars, the maximum shoot recovery after rewarming was recorded for explants of cv. Meteor (89.3%), and the minimum shoot recovery was for those of cv. Skromnitsa (24.2%). The average recovery rate for all subsets was 58.8% (Table 5; Fig. 1). According to the dispersion analysis, the influence of the genotype on the regeneration of shoot tips after thawing was statistically significant ($p = 0.0003$).

The literature reports a significant influence of genotype on shoot regeneration after cryopreservation for

Table 5. Shoot recovery after cryopreservation of 12 red raspberry cultivars (apical shoot tips of *in vitro* plants) on 6th wk after rewarming

Cultivar	Shoot recovery, (%)	
	−LN	+LN
Skromnitsa	96.7 ± 3.3^{ab}	24.2 ± 5.6^{d}
Novokitayevskaya	96.3 ± 3.7^{ab}	40.0 ± 6.5^{cd}
Sputnitsa	95.2 ± 4.8^{ab}	45.5 ± 1.1^{cd}
Kokinskaya	85.0 ± 7.6^{ab}	51.7 ± 1.7^{bcd}
Samarskaya Plotnaya	86.7 ± 6.7^{ab}	55.2 ± 10.3^{bcd}
Barnaulskaya	92.6 ± 7.4^{ab}	56.1 ± 17.3^{bcd}
Progress	82.6 ± 3.8^{abc}	56.8 ± 9.1^{bcd}
Belaya Spirina	100.0 ± 0.0^{a}	57.4 ± 6.3^{bcd}
Shartashskaya	87.0 ± 6.7^{abc}	67.2 ± 11.2^{bc}
Balsam	88.9 ± 5.6^{bc}	80.6 ± 6.3^{bc}
Sokolenok	83.3 ± 3.3^{bc}	81.1 ± 7.0^{bc}
Meteor	100.0 ± 0.0^{a}	89.3 ± 0.7^{b}
Means	$\mathbf{91.2 \pm 1.8}^{bc}$	$\mathbf{58.8 \pm 5.3}^{bcd}$

$n = 60$ explants for '+LN' and 30 explants for '−LN'.

'−LN'—buds were treated with all the solutions but not plunged into liquid nitrogen. '+LN'— buds were treated with all the solutions and were plunged into liquid nitrogen.

Data with the *same letters* are not significantly different ($p < 0.05$).

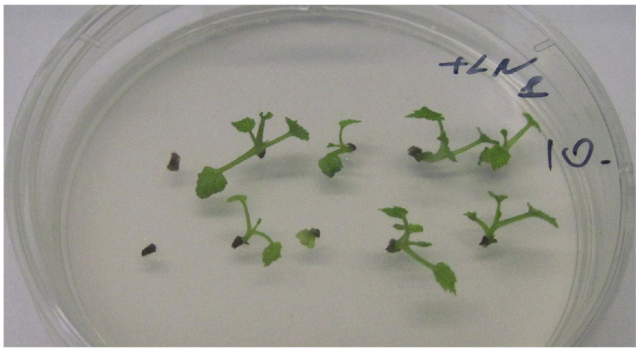

Figure 1. Shoot recovery of apical buds of red raspberry 'Barnaulskaya'—6 wk after rewarming

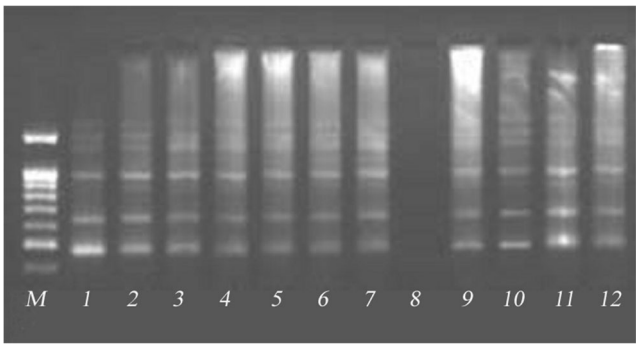

Figure 2. Comparison of electrophoregrams of PCR-products generated with ISSR primer $(GT)_8C$ in initial *in vitro* plant and in 10 microplants recovered after rewarming in red raspberry 'Samarskaya Plotnaya'. M molecular weight marker (100 bp + 1500, http://russia.sibenzyme.com), 1 initial *in vitro* plant used as a source of explants of 'Samarskaya Plotnaya' for cryopreservation, 2–7 and 9–12 plants regenerated after rewarming, 8 H_2O

almost all studies of representatives of different taxa, including *Rubus* accessions with 50–85%, (average 68%) (Wang *et al.* 2005), and 60–100% (average 85%) (Gupta and Reed 2006; Reed 2008). Therefore, the current results of post-cryogenic recovery of red raspberry cultivars were comparable with those obtained by other laboratories and genebanks (Table 1).

Several modifications of the droplet vitrification protocol were applied for the cryopreservation of red raspberry (*Rubus idaeus* L.) accessions (Nukari *et al.* 2011; Condello *et al.* 2011) (Tables 1and 3). It should be noted that the current modified protocol makes it possible to considerably shorten the cryopreservation procedure compared with the original protocol by Panis *et al.* (2005) from 14 to 11 wk.

The other differences between the original droplet vitrification method (Panis *et al.* 2005) and proposed protocol are described in Table 3 in detail along with other droplet vitrification protocols developed previously for cryopreservation of raspberry accessions (Nukari *et al.* 2011; Condello *et al.* 2011).

The current droplet vitrification procedure contained the following modifications:

First, the changed plant growth regulator composition of the micropropagation medium was different from that suggested in the above-cited publications (Table 3).

Second, a lengthy (4-wk-long) pretreatment of the initial microplants on the sucrose-rich (60 g L^{-1}) medium was omitted in contrast to the protocol by Panis *et al.* (2005) (Table 3). Other *Rubus* protocols use different pretreatments. It was shown that 1 wk of cold acclimation (22 °C 8-h day and 1 °C 16-h night) of the initial microplants, and a combination of the cold acclimation with 50 μM ABA, significantly improved regrowth for several *Rubus* genotypes from slow freezing (Reed 2008). Nukari *et al.* (2011) made the pretreatment of initial *Rubus* microplants by cold acclimation and sucrose-rich medium (0.25–0.75 M, 86–256 g L^{-1}) for droplet vitrification. Condello *et al.* (2011) did not use any pretreatment and shortened the total duration of the droplet vitrification protocol (Table 3). However, only one raspberry accession was used in the study. The droplet vitrification modified protocol of Condello *et al.* (2011) was noted when developing the modifications in the present study.

Table 6. Number of amplified bands with ISSR and SCoT primers in initial donor plants and in 10 plantlets regenerated from shoot tips of each red raspberry cultivar following droplet vitrification cryo-procedure

Molecular marker	No. of primers	Raspberry cultivars			
		'Barnaulskaya'		'Samarskaya Plotnaya'	
		No. of bands	No. of polymorphic bands	No. of bands	No. of polymorphic bands
ISSR	$(AG)_8C$	8	0	8	0
	$(GT)_8C$	3	0	6	0
Total	Total	11	0	14	0
SCoT	SCoT4	3	0	8	0
	SCoT12	7	0	0	0
Total	Total	10	0	8	0

Third, the LS medium used from 5–7 h during pretreatment and the osmoprotection stage in the Panis *et al.* (2005) protocol was replaced by incubating the isolated apical buds in the liquid MS hormone-free medium with 30 g L^{-1} of sucrose for 1 h, followed by incubating the explants in the LS medium for 20 min. In contrast to the protocol by Condello *et al.* (2011), incubation was completed in the light instead of darkness (Table 3). As was shown by other authors, the treatment timing in LS and PVS2 vary for different *Rubus* cryopreservation methods. For example, in the encapsulation and vitrification (EV) protocol, the treatment timings are 90 min (LS) and 180 min (PVS2) (Wang *et al.* 2005), whereas in the PVS2-vitrification protocol by Gupta and Reed (2006), the treatment timings are 20 min (LS) and 20 min (PVS2).

Fourth, the droplet vitrification protocol developed by Panis *et al.* (2005) for banana meristems was used with some modifications for cryopreservation of many plant species. These modified droplet vitrification protocols include various recovery media with different plant growth regulators (BA, kinetin, and zeatin) (Reed 2008; Wang *et al.* 2014; Panis *et al.* 2016). In the present study, MS medium with 1.4 μM of zeatin-riboside, 2.9 μM of IAA, and 0.6 μM of GA (Towill, 1983) was used at the final stage of post-cryogenic recovery (Table 3) because zeatin was superior for *in vitro* propagation of *Rubus* accessions compared to other cytokinins (Debnath 2004; Nicuță *et al.* 2014; Zayova *et al.* 2016).

Assessment of the trueness to type in recovered plants Both ISSR primers [$(AG)_8C$, $(GT)_8C$] generated 11 fragments in cultivar Barnaulskaya and 14 fragments in cv. Samarskaya Plotnaya (Fig. 2, Table 6). In total, both SCoT primers [SCoT4, SCoT12] generated 10 fragments in cv. Barnaulskaya. Of the two SCoT primers used, PCR products were obtained only with SCoT4 in the case of cv. Samarskaya Plotnaya (Table 6). The analysis of electropherograms of all fragments did not detect any differences between the DNA bands in the donor microplants and in the 10 post-cryopreservation regenerants of each cultivar (Fig. 2, Table 6). These results corresponded to published data. The majority of research on cryopreservation of apical shoot tips or dormant buds demonstrates stability of the analyzed DNA loci in the post-cryopreservation regenerants (Schäfer-Menuhr *et al.* 1997; Mix-Wagner *et al.* 2002; Keller *et al.* 2006; Kaczmarczyk *et al.* 2010; Castillo *et al.* 2010; Zhang *et al.* 2015; Solov'eva *et al.* 2016; Matsumoto 2017). Polymorphism detected in a few cases (Castillo *et al.* 2010) was due to the initial heterogeneity of the donor microplants, which served as the source of explants for cryopreservation.

Conclusions

The modifications to the droplet vitrification protocol included removal of the initial microplant pretreatment stage. Modified media were used at the stages of initial micropropagation, explant isolation, and post-cryogenic regeneration. The modifications resulted in a reduction in the duration of some cryo-conservation stages compared to the initial protocol by Panis *et al.* (2005), and reduced the total duration from 14 to 11 wk. The modified cryopreservation method developed demonstrated a level of post-cryogenic regeneration for 12 raspberry cultivars that was comparable with the literature. The results of ISSR and SCoT marker analysis confirmed the genetic stability of the analyzed loci of post-cryopreservation regenerants compared to donor *in vitro* plants of two analyzed raspberry cultivars.

VIR will further expand cryopreservation of the raspberry and blackberry collections using the modified droplet vitrification method.

Funding information The paper has been prepared with the support from the Program of Fundamental Research of the State Academies of Sciences (Russian Federation) for 2013–2020.

References

Anonymous (2015) Annual Report of the ICAR-National Bureau of Plant Genetic Resources 2014–2015, ICAR-NBPGR, Pusa Campus, New Delhi, India, 210+x p

Antonova OY, Dunaeva SE, Ukhatova YV, Kamylina NY, Dolganova NA, Lisicyna OV, Gavrilenko TA (2015) *In vitro* improvement of raspberry varieties from raspberry bushy dwarf virus (RBDV) using complex therapy method. Dostizheniya nauki i tekhniki APK 29(7): 61–64 **(in Russian)**

Bologovskaja RP (1949) *Malina* [Raspberry]. Sel'hozgiz Publ, Moscow-Leningrad

Castillo NRF, Bassil NV, Wada S, Reed BM (2010) Genetic stability of cryopreserved shoot tips of *Rubus* germplasm. *In Vitro* Cell Dev Biol–Plant 46:246–256

Catalog of Raspberry cultivars. Available at: strawberryfarm.info/raspberry-sort-45.html. Cited 25 Jan 2017

Collard BCY, Mackill DJ (2009) Start Codon Targeted (SCoT) Polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86

Condello E, Ruzić D, Panis B, Caboni E (2011) Raspberry cryopreservation by droplet vitrification technique. Acta Hortic 918:965–969

Debnath SC (2004) Clonal propagation of dwarf raspberry (*Rubus pubescens* Raf.) through *in vitro* axillary shoot proliferation. Plant Growth Reg 43:179–186

EU GEN RES "European small berries genetics resources" Available at: https://www.bordeaux.inra.fr/genberry/doc/dissemination/genres036/GENRES036-European-raspberry-germplasm-list.pdf Cited 25 Jan 2017

Gavrilenko T, Antonova O, Shuvalova A, Krylova E, Alpatyeva N, Spooner D, Novikova L (2013) Genetic diversity and origin of

cultivated potatoes based on plastid microsatellite polymorphism. Genet Resour Crop Evol 60:1997–2015

Graham J, McNicol RJ, Greig K, Van de Ven WTG (1994) Identification of red raspberry cultivars and an assessment of their relatedness using fingerprints produced by random primers. J Hort Sci 69: 123–130

Gupta S, Reed BM (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. Cryo-Letters 27:29–42

Kaczmarczyk A, Houben A, Keller ER, Mette MF (2010) Influence of cryopreservation on the cytosine methylation state of potato genomic DNA Cryo-Letters 31(5):380–391

Kaczmarczyk A, Rokka V-M, Keller ERJ (2011) Potato shoot tip cryopreservation. A review. Potato Res 54:45–79

Keller ERJ, Senula A, Leunufna S, Grűbe M (2006) Slow growth storage and cryopreservation—tools to facilitate germplasm maintenance of vegetatively propagated crops in living plant collections. Int J Refrig 29:411–417

Kovalchuk I, Turdiev T, Kushnarenko S, Rakhimbaev I, Reed BM (2010) Cryopreservation of raspberry cultivars: testing techniques for long-term storage of Kazakhstan's plant germplasm In: Turuspekov Y (Ed) Khazakhstan Plant Science and Biotechnology.The Asian Aust J Plant Sci Biotech 4(Special Issue 1):1–4

Kovalchuk IY, Turdiev TT, Uspanova GK, Madiyeva GA, Chukanova NI, Reed BM (2014) Cryopreservation and cold storage of fruit, berry crops and grape germplasm in Kazakhstan. In: Proceedings Plant biology and biotechnology international conference May 28-30, Almaty, Kazakhstan: P. 43

Lamoureux D, Sorokin A, Lefèvre I, Alexanian S (2011) Investigation of genetic diversity in Russian collections of raspberry and blue honeysuckle. Plant Genet Resour 9(2):202–205

Lefèvre I, Ziebel J, Guignard C, Sorokin A, Tikhonova O, Dolganova N, Hoffmann L, Eyzaguirre P, Hausman J-F (2011) Evaluation and comparison of nutritional quality and bioactive compounds of berry fruits from *Lonicera caerulea*. *Ribes* L species and *Rubus idaeus* grown in Russia J Berry Res 1:159–167

Matsumoto T (2017) Cryopreservation of plant genetic resources: conventional and new methods. Rev Agric Sci 5:13–20

Matsumoto T, Sakai A, Yamada K (1994) Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia Japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Rep 13:442–446

Mix-Wagner G, Schumacher HM, Cross RJ (2002) Recovery of potato apices after several years of storage in liquid nitrogen. Cryo-Letters 24:33–41

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nicuță D, Rotilă G, Ciobanu Ș (2014) Aspects regarding the *in vitro* multiplication of the *Rubus hirtus* L. species. Studii şi Cercetări Biologie 23(1):79–84

Nukari A, Uosukainen M (2007) Cryopreservation in the Finnish national germplasm programme for horticultural plants. Adv Hort Sci 21(4): 232–234

Nukari A, Uosukainen M, Laamanen J, Rantala S (2011) Cryopreservation of horticultural plants at MTT. In: Grapin A, Keller ERJ, Lynch PT, Panis B, Revilla Bahillo A, Engelmann F (eds) Proceeding of the final meeting COST Action 871 CryoPlanet "Cryopreservation of crop species in Europe". pp. 93-97

Nukari A, Uosukainen M, Rokka V-M, Antonius K, Wang Q, Valkonen JPT (2009) Cryopreservation techniques and their application in vegetatively propagated crops in Finland. Agric Food Sci 18:117–128

Panis B, Piette B, Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Sci 168:45–55

Panis B, Van den Houwe I, Swennen R, Rhee J, Roux N (2016) Securing plant genetic resources for perpetuity through cryopreservation. Indian J Plant Genet Resour 29(3):300–302

Reed BM (2008) Cryopreservation of temperate berry crops. In: Reed BM (ed) Plant cryopreservation: A practical guide. Springer, New York, pp 333–364

Reed BM, DeNoma J (2012) Tissue Culture and Cryopreservation. In: Hummer K (ed) Corvallis repository annual report for 2012. United States Department of Agriculture, https://iapreview.ars.usda.gov/SP2UserFiles/Place/20721500/AnnualReports/CorvallisAnnualReport2012.pdf, pp 29. Cited 05 Apr 2017

Reed BM, Engelmann F, Dulloo ME, Engels JMM (2004) Technical guidelines for the management of field and *in vitro* germplasm collections. Handbooks for Genebanks No. 7. Rome, IPGRI

Sakai A (1997) Potentially valuable cryogenic procedures for cryopreservation of cultured plant meristems. In: Razdan MK, Cocking EC (eds) Conservation of plant genetic resource *in vitro*. Science Publishers, USA, pp 53–66

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nuclear cells of navel orange (*Citrus sinensis* Osb. var.*brasiliensis* Tanaka) by vitrification. Plant Cell Rep 9:3–33

Schäfer-Menuhr A, Schumacher HM, Mix-Wagner G (1997) Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid nitrogen. Plant Genet Resour Newsl 111:19–24

Solov'eva AI, Vysotskaya ON, Dolgikh YI (2016) Effect of dehydration duration of apices on characteristics of *in vitro* plants of *Fragaria vesca* after cryopreservation. Russ J Plant Physiol 63(2):243–251

Towill LE (1983) Improved survival after cryogenic exposure of shoot tips derived from *in vitro* plantlet cultures of potato. Cryobiology 20: 567–573

Vysotskaya ON, Popov AS (2005) Method for cryogenic *in vitro* keeping of meristems isolated from red raspberry plants. Patent RF, no. 2248121 http://russianpatents.com/patent/224/2248121.html Cited 25 Jan 2017

Wang B, Wang RR, Cui ZH, Bi WL, Li JW, Li BQ, Ozudogru EA, Volk GM, Wang QC (2014) Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. Biotechnol Adv 32:583–595

Wang LY, Li YD, Sun HY, Liu HG, Tang XD, Wang QC, Zhang ZD (2017) An efficient droplet-vitrification cryopreservation for valuable blueberry germplasm. Sci Hortic 219:60–69

Wang QC, Laamanen J, Uosukainen M, Valkonen JPT (2005) Cryopreservation of *in vitro* grown shoot tips of raspberry (*Rubus idaeus* L.) by encapsulation-vitrification and encapsulation-dehydration. Plant Cell Rep 24:280–288

Zayova E, Stancheva I, Geneva M, Petrova M, Dimitrova L (2016) Comparison of antioxidant activity of the fruits derived from *in vitro* propagated and traditionally cultivated tayberry plants. J Sci Food Agric 96:3477–3483

Zhang Z, Skjeseth G, Elameen A, Haugslien S, Sivertsen A, Clarke L, Wang Q-C, Blystad DR (2015) Field performance evaluation and genetic integrity assessment in *Argyranthemum* 'Yellow Empire' plants recovered from cryopreserved shoot tips. *Vitro* Cell Dev Biol– Plant 51(5):505–513