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ORIGINAL ARTICLE

Droplet vitrification technique for cryopreservation of a large diversity of blackcurrant (*Ribes nigrum* L.) cultivars

Saija Rantala[1,2] · Janne Kaseva[3] · Anna Nukari[4] · Jaana Laamanen[5] · Saara Tuohimetsä[1] · Saila Karhu[6] · Merja Veteläinen[7] · Hely Häggman[2]

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Abstract

The aim of plant gene banks is to preserve genetic resources selected based on their phenotypic, agronomic, historical or other cultural values for future utilization. In the present study the modified PVS2 droplet vitrification technique was tested and optimized for cryopreservation of a large diversity of blackcurrant (*R. nigrum* L.) accessions propagated in vitro and selected into a national gene bank core collection. Out of four accessions tested to optimize the method, three recovered and regenerated by 89–97% on average, but one recalcitrant in vitro line only by 25%. The tested post-cryopreservation recovery media with different macronutrient and growth regulator levels showed no generalized effect on regenerated shoots, but the effect of recovery media was different between cultivars. When the whole regeneration chain from cryopreservation via micropropagation to greenhouse conditions was tested, shoots at least 1 cm in length were found necessary for successful transfer ex vitro. The long-term cryopreservation of 22 blackcurrant accessions was finally conducted, with practices slightly modified from the tested protocol. The estimated recovery of shoot tips after 9 weeks in vitro was 17–94% with at least 75% recovery in seven accessions and at least 40% recovery in 19 out of 22 accessions. Only one accession had no cryopreservation success. The results demonstrated that the modified droplet vitrification technique is appropriate for a large diversity of blackcurrant accessions. However, cultivar-related differences and recovery procedures are to be considered for success in regeneration and ex vitro adaptation.

Key message

The modified droplet vitrification method provides a feasible tool for conservation of plant genetic resources of blackcurrant.

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✉ Saija Rantala
saija.rantala@luke.fi

[1] Production Systems, Natural Resources Institute Finland (Luke), Survontie 9 A, 40500 Jyväskylä, Finland

[2] Faculty of Science, Ecology and Genetics Unit, University of Oulu, P.O.Box 3000, 90014 Oulu, Finland

[3] Natural Resources, Natural Resources Institute Finland (Luke), Tietotie 4, 31600 Jokioinen, Finland

[4] Production Systems, Natural Resources Institute Finland (Luke), Latokartanonkaari 9, 00790 Helsinki, Finland

[5] Natural Resources, Natural Resources Institute Finland (Luke), Survontie 9 A, 40500 Jyväskylä, Finland

[6] Production Systems, Natural Resources Institute Finland (Luke), Itäinen Pitkäkatu 4 A, 20520 Turku, Finland

[7] Boreal Plant Breeding Ltd., Myllytie 10, 31600 Jokioinen, Finland

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Introduction

The aim of the conservation of food plant genetic resources is to secure their future use for breeding, cultivation and nutrition purposes. In addition, conservation of plant genetic resources is carried out in order to ensure their future adaptability (Ford-Lloyd and Jackson 1986). Thus, for within species conservation it is important to cover the range of diversity within and between different origins and geographical regions. Blackcurrant (*Ribes nigrum* L.) is a woody shrub native to Northern Europe and well appreciated by its high economic importance due to the aromatic berries and leaves. Active breeding and domestication have resulted in a high diversity of commercial varieties and local races, all called cultivars in this article. Blackcurrant fruits are valued for their high nutritional and dietary qualities e.g. large amounts of vitamins (especially vitamin C), minerals, and antioxidant compounds (Brennan and Graham 2009; Vagiri et al. 2016).

Cryopreservation i.e. cryogenic preservation in liquid nitrogen (− 196 °C or close to that) is a useful conservation tool referring to storage of plant cells, tissues and organs at ultra-low temperature. Generally, cryopreservation guarantees the viability and genetic integrity of plant material for a long period of time, through its ultra-low temperature (e.g. Prudente and Paiva 2017). A wide range of protocols are available for many plant species and different types of plant material (Reed 2008). Cryopreservation techniques have been employed for the conservation of many economically important fruit species (Benelli et al. 2013) and ornamental species (Kulus and Zalewska 2014) but also for endangered species like *Rubus humulifolius* (Edesi et al. 2020). For instance, the droplet vitrification technique with different modifications has been used successfully to cryopreserve e.g. *Allium* (Volk et al. 2004), *Musaceae* (Panis et al. 2005), *Rosa* (Pawlowska and Szewczyk-Taranek 2014) and *Rubus idaeus* (L.) (Ukhatova et al. 2017).

Cryopreservation of blackcurrant has been studied for a long time using a variety of cryopreservation methods and protocols (Benson et al. 1996; Reed et al. 2001, 2004, 2005; Johnston et al. 2007; Harding et al. 2009; Green and Grout 2010; Rantala et al. 2019). Different cryopreservation techniques are employed for *Ribes* species in USDA-ARS National Plant Germplasm System, Plant and Animal Genetic Resources Preservation Unit, Fort Collins, CO (Jenderek and Reed 2017). However, the transfer of existing protocols between laboratories and their successful implementation in practical conservation work of germplasm and for different genotypes requires precision (Reed et al. 2004; Reed 2017). Maintaining many different methods, especially in small laboratories, can be challenging and, therefore, to conserve large number of accessions, robust and easy protocols are needed (Panis et al. 2005; Reed et al. 2005).

In Europe, the genetic resources of blackcurrant selected for long-term preservation in gene banks are mainly maintained in field collections. Due to the high risk to lose usability of collections mainly due to adverse weather conditions and risk of pests and diseases like black currant reversion virus (BRV), there are severe needs to ensure long-term conservation through more advanced methodologies. One solution is the implementation of suitable cryopreservation technique for blackcurrant ex situ conservation. The Finnish national core collection of blackcurrant cultivars, including both black and green berry types, was selected as part of the multinational project RIBESCO (Antonius et al. 2012; Karhu et al. 2012). The aim of the core collection establishment was to capture the entire range of genetic variation based on values and diversity defined by the genotypic and phenotypic characterisation, and by agronomic, historical or other cultural values. The core collection is managed by The Finnish National Genetic Resources Programme for Agriculture, Forestry and Fishery.

The aim of the present study was to optimize the modified droplet vitrification technique for cryopreservation of Northern European blackcurrant accessions and to test its suitability for a large diversity of cultivars selected for Finnish core collection of *Ribes*. The post-cryopreservation recovery of shoot tips and regrowth of microshoots in vitro were evaluated, and viability of microplants of four cultivars was first tested in vivo. In addition, the effect of the recovery medium, used after thawing on recovery of shoot tips was evaluated. Finally, the suitability of the tested protocol for long-term cryopreservation of 23 blackcurrant cultivars was examined.

Materials and methods

Plant material

In the present study, in vitro cultures of altogether 27 blackcurrant cultivars were used as source material for cryopreservation. In vitro cultures of five blackcurrant cultivars were first used to optimize and evaluate the selected cryoprotocol i.e. the modified droplet vitrification technique. Thereafter altogether 23 blackcurrant cultivars were cryopreserved with this optimized method. In vitro cultures were initiated from donor plants maintained in insect-proof greenhouse conditions in Laukaa (Finland) or in a field collection in Piikkiö (Finland) at Natural Resources Institute Finland (Luke). All

plants maintained in greenhouse were originally propagated via micropropagation and tested to be free from blackcurrant reversion virus. The in vitro cultures of field-maintained cultivars were initiated from cuttings taken from donor plants and tested for blackcurrant reversion virus. Before initiation of in vitro cultures, cuttings got thermotherapy from 12 to 21 days at + 37 °C. Only initiations made from cuttings that were tested to be free from blackcurrant reversion virus were selected for further cultivation and used for cryopreservation.

Establishment of in vitro cultures

To establish in vitro cultures, several initiations were made from each cultivar and two to four best ones per cultivar were chosen for further cultivation. At initiation stage, explants were cultured in test tubes on Medium 1 (M1) containing WPM culture media (Lloyd and McCown 1980) supplemented with 6.84 µM zeatin and 20.0 g l^{-1} sucrose adjusted to pH 5.2 before autoclaving and solidified with the mixture of agars (4.375 g l^{-1}Scharlau Agar and 4.375 g l^{-1} Carl Roth Agar). At multiplication stage, shoot cultures were subcultured at 3-week intervals on Medium 2 (M2) containing semisolid G basal medium (Uosukainen 1992) supplemented with 3.33 µM 6-benzyladenine and 20.0 g l^{-1} fructose. The medium was solidified with agars as described above and adjusted to pH 5.0 before autoclaving. Shoot cultures were maintained in the growth room at + 22 ± 1 °C under a 16 h photoperiod with light intensity being on average 79 µmol m^{-2} s^{-1} provided by fluorescent tubes (Osram L 36 W/830 Lumilux warm white, Germany). 3 weeks before the excision of shoot tips, shoot cultures were transferred onto Medium 3 (M3), which was a modified G basal medium with half-strength macronutrients supplemented with 0.44 µM BA and 20.0 g l^{-1} glucose.

The applicability of modified droplet vitrification method for blackcurrant cultivars

Several preliminary studies were first performed to optimize the modified droplet vitrification method by Nukari and Uosukainen (2007) based on Panis et al. (2005), to be suitable for cryopreservation of blackcurrant cultivars. The main modification of the protocol by Nukari and Uosukainen (2007) compared to protocol by Panis et al. (2005), is to freeze the shoot tips on aluminum foil strips inside the closed cryovials, which does not provide direct immersion to liquid nitrogen nor immediate fast freezing. The focus of the preliminary studies (data not shown) was on the cold acclimation of shoot cultures and on the use of plant vitrification solution (PVS2, Sakai et al. 1990) in treatment of shoot tips excised from shoot cultures. The selected protocol was tested with five blackcurrant cultivars: Mortti, Öjebyn, Hedda, Marski, and Mikael (Table 1). Norwegian cultivar Hedda did not belong to the Finnish core collection but was selected for testing especially because, based on our earlier experience, it was recalcitrant to cultivation in vitro.

The cultivars shown in Table 1 were cryopreserved in four independent experimental occasions (replications, called sets in this paper) to repeat the experiment and to get enough shoot tips for long-term cryopreservation. Two shoot cultures (initiation lines) per cultivar of cvs. Mortti, Hedda and Öjebyn were cryopreserved, and in the case of cvs. Mikael and Marski four shoot cultures per cultivar were cryopreserved. In each experiment of those five cultivars, 60 shoot tips, i.e. six cryovials with ten shoot tips in each, were cryopreserved and ten shoot tips were used as non-cryopreserved controls. Half of the cryopreserved shoot tips were used for viability assessment and half for long-term preservation to build up a national germplasm collection.

Before the excision of shoot tips, the in vitro shoot cultures were cold acclimated for 1 week in alternating diurnal temperatures: 8 h in a growth room at + 22 ± 1 °C under

Table 1 Blackcurrant cultivars used in method testing of the optimized cryopreservation protocol

Cultivar	Ancestors and origin
Öjebyn	Selected local strain from North Sweden; commercial cultivar in Nordic countries[1]
Hedda	Crossing between Öjebyn and Melalahti; Norwegian cultivar[1]
Mortti	Crossing between Öjebyn and Wellington XXX; finnish cultivar[2]
Marski	Crossing between Hedda and Mortti; finnish cultivar[1]
Mikael	Crossing between two selections originating both from three successive self-pollinations of the Finnish local variety Brödtorp; finnish cultivar[3]

[1]htpp://www.ribes-rubus.gf.vu.lt/download.htm

[2]Hietaranta and Hiirsalmi (1990)

[3]Information from the archives of Natural Resources Institute Finland (Luke)

light (79 µmol m^{-2} s^{-1}) and 16 h in cold in a refrigerator at + 3 ± 1 °C in darkness, but during the weekend the jars were kept in refrigerator only. Seventy shoot tips (1.5–3 mm in length) were excised from shoot cultures and pre-cultured for 2 days on semisolid MS medium (Murashige and Skoog 1962) supplemented with 0.25 M sucrose and 2.5 g l^{-1} active charcoal under cold acclimation conditions.

Pre-cultured shoot tips were treated in liquid MS medium containing 2 M glycerol and 0.4 M sucrose for 30 min, followed by transfer with sterile sieve into PVS2 (Sakai et al. 1990) for dehydration. Instead of MT medium used in the original PVS2 solution, PVS2 containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide dissolved in liquid MS medium with 0.4 M sucrose was used. Both treatments were done at room temperature and the bottom of the sieve was wiped with sterile filter paper between treatments. Shoot tips were soaked in PVS2 for 20 min and thereafter moved on PVS2 droplets (10 µm) placed on seven aluminum foils (7 mm × 27 mm). Each foil contained three droplets, and ten shoot tips (i.e. three or four shoot tips per droplet) were placed on each foil. The transfer of 70 shoot tips one by one to the PVS2 droplets took from five to nearly 8 min, so the total incubation time of shoot tips in PVS2 was determined to be 28 min. When 28 min incubation time was completed, foils with droplets were placed in empty 1.8 ml cryovials (Sarstedt), which were then sealed with caps and immersed in liquid nitrogen. Shoot tips were remained in PVS2 droplets and droplets on aluminum foils by surface tension. The vials were handled gently, to avoid shoot tips sliding off the aluminum foils. Control shoot tips not to be cryopreserved were rinsed for 30 min in 2 ml of liquid MS medium containing 1 M sucrose, pipetted into non-cryopreserved cryotubes. Control shoot tips were cultivated 9 weeks in vitro like cryopreserved shoot tips described below, but regenerated shoots were not transferred in vivo. Cryopreserved shoot tips were stored in the gas phase of liquid nitrogen in a cryotank (MVE 1520 Eterne with TEC 2000 system monitor, CHART / MVE Applied Technologies, Biological Systems, Minnesota).

Post-thaw recovery of blackcurrant shoot tips in vitro

To evaluate the post-thaw recovery of shoot tips, altogether 12 cryovials (three from each experimental occasions or cryopreserved set) of cultivars Mortti, Öjebyn, Hedda and Marski, were thawed 18 to 20 weeks after the beginning of cryostorage. Cryovials were thawed in a water bath at 40 °C for 3 min. Shoot tips were rinsed in cryovials for 30 min with 2 ml liquid MS medium containing 1 M sucrose. Rinsed shoot tips were first quickly transferred onto sterile filter paper and thereafter placed on Petri dish (10 pcs/plate) on Medium 4 (M4) containing M3 medium supplemented with 0.29 µM GA_3. Plates were kept in the growth room at + 22 ± 1 °C covered first with an aluminium foil for 5 days and then with gauze to keep them first in dark and then in dim light. 1 week after thawing shoot tips were exposed to 16 h photoperiodic light as described above and transferred onto new plates containing M2 medium for 2 weeks. Thereafter shoot tips were subcultured at 3 weeks interval on M2 medium using first 100 ml Erlenmeyer flasks and then 300 ml glass jars as culture vessels with lids. The recovery of shoot tips was evaluated at 3 weeks intervals and shoot tips that produced at least one viable shoot were interpreted as recovered. Cryovial-specific recovery rates were calculated based on the number of recovered shoot tips and the number of cryopreserved shoot tips in a cryovial.

Transfer of regenerated in vitro shoots into in vivo conditions

To evaluate the efficiency of the regeneration chain, the shoots recovered after cryopreservation were transferred in vivo. Shoots of cv. Mortti grew well in vitro and they were planted in vivo 9 weeks after thawing. Shoots of cvs. Hedda, Öjebyn and Marski were planted after 12 weeks in vitro. Before planting, shoot clumps were divided into single shoots and shoots were graded by length to categories of 0.5 cm, 1 cm or ≥ 1.5 cm. To promote the growth of roots, the basal parts of the shoots were dipped in indole-3-butyric acid potassium salt (100 mg/l, KIBA Sigma and 50 mg/l H_2BO_2) before planting. Shoots were planted in propagator boxes filled with the mixture of peat (containing 80% peat, 10% sand and 10% perlite), soil and sand (10:5:3). In the cases of cvs. Mortti, Marski and Öjebyn, four boxes were used for each cultivar, but the shoots of cv. Hedda were planted in two boxes due to the low number of shoots. Propagator boxes were placed first into the growth room at + 19 °C with a 16 h photoperiod provided by fluorescent tubes (Sylvania Gro lux F36W/GRO T8, Germany). Shoots were first watered as needed by using a spray or dropper bottle, and after 5 to 7 weeks of acclimatization, propagator boxes were transferred into standard greenhouse conditions at + 20/ + 18 °C (day/night) with automated lighting and irrigation. The survival and the quality of viable plants were evaluated 13 weeks after planting. Plants were graded first with the six–grade scale, but in statistical analyses the grades were reclassified from scale of zero–five to a binary scale (dead and poor shoots vs. viable shoots).

The impact of recovery medium

Because of the poor recovery of thawed shoot tips of cv. Hedda, the remaining twelve cryovials of cvs. Hedda and Mikael were thawed to test the effect of culture medium used after thawing on the recovery of shoot tips. Shoot tips of both cultivars were placed either onto tissue culture media M2, M3 or M4 for 1 week and thereafter cultivated

as described above on M2 medium. The recovery of shoot tips was evaluated 13 weeks after thawing by recording their survival and number of the regenerated shoots. The amount of clear blackening and visible hyperhydricity (Debergh et al. 1992) of shoots was also observed. In vitro cultivation of cv. Hedda microshoots was continued on M2 medium three more weeks (in total 16 weeks) prior to transfer of shoots in vivo. The length of the shoots was measured and graded and shoots ca. 1 cm–1.5 cm in length were selected for transplanting. Up to 60 highest shoots from three vials per treatment were transferred in vivo. The viability of shoots was evaluated 13 weeks after transplanting in vivo. Shoots of cv. Mikael were not transferred in vivo.

Cryopreservation of blackcurrant core collection cultivars for long-term preservation

The cryopreservation of 23 blackcurrant cultivars for long-term preservation was conducted following the practises described above. With a few exceptions, cultivars were cryopreserved in three separate occasions (replications, called sets in this paper) and on each occasion 80 shoot tips were cryopreserved. In each set, the target was to cryopreserve five cryovials in the cryotank for long-term conservation whereas three vials were thawed for viability assessment after at least one-hour exposure into liquid nitrogen, each vial with ten shoot tips. In some occasions the number of the excised shoot tips was different than 80 and therefore the number of vials thawed or stored per occasion could vary. However, the cryopreservation of cv. Hedda was repeated only twice because it had not been selected into the core collection of *Ribes* in Finland. The size of the shoot tips excised from shoot cultures varied slightly between accessions and cryopreservation sets due to the differences of in vitro cultures. In most cases the transfer of shoot tips was started after soaking in PVS2 for 15 min, so it was possible to use up to 13 min for the transfer of shoot tips into PVS2 droplets. However, the total incubation time of shoot tips in PVS2 before cryopreservation varied slightly with range of 25–36 min. Shoot tips thawed for viability assessment were placed on M3 medium for 1 week and thereafter cultivated on M2 medium. The recovery of shoot tips was evaluated 6 and 9 weeks after thawing, to find out the proper recovery time needed per cultivar for recovery evaluation. The data of cv. Melalahti were not tested statistically due to difficulties in in vitro culture and the poor recovery results of the thawed shoot tips. Besides testing the post-cryopreservation success of cultivars, the results were used to estimate the mean variation in recovery results between the cryopreserved sets within cultivars and between thawed cryovials within sets.

Statistical analysis

The mean recovery of the cryopreserved shoot tips after 6 and 9 weeks for cvs. Mortti, Öjebyn, Hedda and Marski were compared using generalized linear mixed model (GLMM). The interaction of week and cultivar was included as fixed effect. Correlation between weeks was taken into account through random effect. Cryovials from the same cryopreserved set and shoot culture (initiation line), from which the shoot tips were excised, was used as random effects. Binomial distribution with logit link was used to analyze the recovery rate, and estimated means were backtransformed to the original scale. The difference between control and cryopreserved shoot tips was tested by the same model, where the effect of cultivar was replaced by the control. The estimated recovery rates of cultivars cryopreserved for long-term preservation were analysed with the same GLMM model as the previous shoot tips. Means with confidence intervals (Cl) or standard deviations of variations between cryopreserved sets and thawed cryovials per set were calculated to every cultivar.

The effect of recovery media on recovery of shoot tips was compared by a generalized linear model (GLM). Recovery medium used after thawing (M2, M3 and M4) and cultivars (Hedda and Mikael), and their interactions, were used as fixed effects. Binomial distribution with logit link was used to analyze the recovery rate. In recovery media test, GLMMs were used to compare the number of shoots gained from regenerated shoot tips and amount of hyperhydricity and blackening detected in regenerated shoots or rosettes. In addition to previous GLM, random effects of cryopreserved sets and interaction of treatment and cryopreserved sets were used. Negative binomial distribution with log link was used to analyze the regenerated shoots and ordered multinomial distribution with cumulative logit link to hyperhydricity and blackening, respectively.

The viability of shoots in vivo was analyzed using shoot length (0.5, 1 and 1.5 cm), cultivar (Öjebyn, Marski and Mortti) and their interaction as fixed effects. Correlation between cryovials thawed from same cryopreserved sets was taken into account in random effect. Binary distribution with logit link was used to analyze the viability rate. Simplified version of the same model, using only treatment (i.e. recovery media used in vitro culture) as fixed effect, was used to compare the viability of cv. Hedda shoots in vivo.

Maximum likelihood (ML) or residual pseudo likelihood (RSPL) or Laplace estimation methods were used, and the degrees of freedom were calculated using the Residual or the Kenward-Roger method, respectively. The method of

Westfall was used for pairwise comparisons of means (Westfall 1997). A significance level of $\alpha = 0.05$ was used in all the analyses conducted. The analyses were performed using the GLIMMIX procedure of the SAS Enterprise Guide 7.15 (SAS Institute Inc., Cary, NC, USA).

Results

The post-cryopreservation recovery of shoot tips in vitro

After 9 weeks in vitro, the recovery of shoot tips was on average 87.5% (76.8–93.6%) and 96.5% (87.3–99.1%) for cryopreserved and non-cryopreserved control shoot tips ($p = 0.128$), respectively. However, the recovery of cryopreserved shoot tips differed ($p < 0.001$) between cultivars (Table 2). The recovery of shoot tips was significantly lower for cv. Hedda than for cvs. Mortti, Marski or Öjebyn, but between those three cultivars differences were not significant (Table 2). Both cryopreserved and control shoots of cv. Hedda were tiny and suffered blackening of leaves. Yet the recovery result of four non-cryopreserved cryovials of cv. Hedda ranged between 60–100%. The recovery of non-cryopreserved shoot tips was 100% for cv. Mortti and Öjebyn and ranged between 78–100% for cv. Marski.

Over the cultivars, there was no significant difference in the number of cryopreserved explants defined as regenerated ones between the evaluations after 6- and 9-week cultivation in vitro ($p = 0.085$). In cvs. Marski, Mortti and Öjebyn, the recovery of cryopreserved shoot tips improved from the week 6 to the week 9, but the difference was significant only for cv. Marski ($p = 0.033$). In cv. Hedda, the number of explants defined as regenerated ones decreased from week 6 to the week 9, because the viability of some regenerated shoots decreased during in vitro culture. In addition to cv. Hedda, the decrease in viability during in vitro culture was observed only for some shoots of cv. Öjebyn. In the case of cvs. Öjebyn, Hedda and Marski, the extension of post-cryopreservation recovery time in vitro up to 12 weeks enhanced recovery results only for two vials of cv. Öjebyn, and one vial of cv. Hedda. The number of 1.5 cm long shoots gained per one cryovial, each containing ten shoot tips, was on average 3.5, 4.8 and 7.3 for cvs. Öjebyn, Marski and Mortti, respectively (Table 2). The cv. Hedda produced only tiny shoots.

The variation in the recovery results between four cryopreserved sets was 3%, 8%, 10% and 12% for cvs. Mortti, Marski, Hedda and Öjebyn, respectively ($p = 0.351$). The over-sets median of variation in recovery results for cultivars was 8%. The number of contaminations in protocol testing was low, only one cryovial of cv. Marski and one cryovial of cv. Hedda thawed for recovery testing were lost due to contamination during in vitro culture.

The recovery medium used after thawing affected differently on the recovery of shoot tips of cvs. Hedda and Mikael ($p = 0.040$) as shown in Table 3. In cv. Hedda, shoot tips placed on M4 medium were weaker compared to those on M3 medium ($p = 0.010$). However, there were no significant differences in the recovery of shoot tips placed first either onto M3 medium or onto M2 medium ($p = 0.337$). The difference between the media M2 and M4 was neither significant ($p = 0.110$). The first recovery medium used after thawing did not affect the recovery of the shoot tips of cv. Mikael.

The amount of hyperhydricity found on recovered shoots differed according to the culture medium used after thawing ($p = 0.020$). In both cv. Hedda and cv. Mikael, the hyperhydricity of leaves was more common among shoots regenerated from shoot tips placed first onto M3 medium than on the other media, but the difference was significant only in the case of Mikael between M2 and M3 medium ($p = 0.036$) (Table 4). The first culture medium used after thawing also

Table 2 The mean recovery of the cryopreserved shoot tips after 9 weeks of in vitro culture and average number of shoots gained per vial for cvs. Hedda, Öjebyn, Marski and Mortti. The number of regenerated shoots was counted after 9 weeks of in vitro culture for cv. Mortti and after 12 weeks of in vitro culture for other cultivars

Cultivar	Vials thawed	Recovery %		Average number of regenerated shoots per vial		
				Size category		
	N	Mean	Cl	0.5 cm	1 cm	≥ 1.5 cm
Hedda	12	25.5[b]	12.8–44.4	6.8	0	0
Öjebyn	12	88.7[a]	75.3–95.2	12.3	5.3	3.5
Marski	11*	95.5[a]	87.0–98.5	10.0	8.3	4.8
Mortti	12	97.0[a]	90.3–99.1	4.0	5.0	7.3

Cl 95% confidence intervals

N Number of thawed cryovials with ten shoot tips in each

Different superscript letters indicate significant differences ($p < 0.05$) in estimated recovery rate of shoot tips in vitro between cultivars.

*Shoot tips from one cryovial of cv. Marski were excluded from the experiment due to contamination during in vitro culture.

had different effect on blackening of regenerated shoots in cv. Hedda than in cv. Mikael ($p < 0.001$), the latter cultivar being more prone to shoot blackening. In cv. Hedda, blackening of leaves was most common among shoots, which were regenerated from shoot tips placed onto M4 medium after thawing whereas in cv. Mikael the blackening of leaves was less pronounced (Table 4.) The first regeneration medium had different effect on the mean of the shoots produced by regenerated shoot tips ($p = 0.065$). In cv. Hedda, the mean of the shoots was lowest for shoot tips placed first onto M4 medium (Table 4.) In cv. Mikael, the tissue culture medium used after thawing did not affect the mean of shoots produced by regenerated shoot tips.

The viability of shoots in vivo

The viability of the shoots in vivo was evaluated 13 weeks after transplanting. At that time there were only three shoots of cv. Hedda alive and therefore cv.Hedda was left out from statistical tests. The viability of the shoots of other cultivars in vivo was affected by the planting size of the shoots ($p < 0.001$) and the cultivar ($p < 0.001$).

Transplanted shoots 0.5 cm in length had lower probability ($p < 0.001$) to stay viable than shoots scored to be 1 cm or 1.5 cm long at the time of planting (Table 5). However, in cvs. Öjebyn and Mortti there was no difference in viability between shoots planted as 1 cm or ≥ 1.5 cm, but in cv Marski there was difference in viability between those planting sizes

Table 3 The estimated probabilities of cryopreserved shoot tips to recover for cvs. Hedda and Mikael as evaluated 13 weeks after thawing. The shoot tips were cultured 1 week on M2, M3 or M4 medium and thereafter 12 weeks on M2 medium. Shoot tips from one plate of cv. Hedda were excluded from the experiment due to a contamination during in vitro culture

First recovery medium	Recovery %					
	cv. Hedda			cv. Mikael		
	N	Mean	Cl	N	Mean	Cl
M2	4	72.5[a,b]	60–100	4	72.5[a]	50–90
M3	4	87.5[a]	80–90	4	78.1[a]	70–90
M4	3	43.3[b]	10–60	3	76.9[a]	69–90

Cl 95% confidence intervals

N number of plates, with ten shoot tips per plate

Different superscript letters indicate significant differences ($p < 0.05$) between estimated recovery rates between the first culture media used.

Table 4 The estimated mean number of shoots per recovered shoot tip of cvs. Hedda and Mikael according to the used recovery medium and the estimated probabilities of hyperhydricity or blackening of the leaves

First recovery medium	Shoots with visible hyperhydricity %		Shoots with clear blackening %		Number of shoots per recovered shoot tip	
	cv. Hedda	cv. Mikael	cv. Hedda	cv. Mikael	cv. Hedda	cv. Mikael
M2	3	6[a]	3.2	47[a]	3.4[a,b]	3.3
M3	8	33[b]	10.5	42[a]	3.8[a]	3.5
M4	6	8[a,b]	18.5	9[b]	2[b]	3.4

Different superscript letters indicate significant differences ($p < 0.05$) in estimated variables between the first culture media used.

Table 5 The estimated probabilities for shoots to be viable after 13 weeks in vivo based on the results from twelve vials of shoots of cvs. Mortti and Öjebyn and eleven vials of cv. Marski

Shoot length score	Viability %							
	cv. Öjebyn		cv. Marski		cv. Mortti		All three cultivars	
	Mean	CI	Mean	CI	Mean	CI	Mean	CI
0.5 cm	12[a]	6–20	32[a]	19–47	69[a]	50–84	34[a]	26–44
1 cm	48[b]	28–68	60[b]	44–75	95[b]	76–99	75[b]	60–86
≥ 1.5 cm	54[b]	39–68	80[c]	68–89	93[b]	87–96	80[b]	73–85

Cl 95% confidence intervals

Different superscript letters indicate significant differences ($p < 0.05$) between length scores in estimated viability of shoots in vivo.

(p = 0.031) (Table 5). Overall, the viability of shoots was 34% (Cl 24–47), 59% (Cl 46–70) and 89% (Cl 78–95) for cvs. Öjebyn, Marski and Mortti, respectively.

The number and length of shoots of cv. Hedda transplanted in vivo from the recovery medium test were not equal because of the shortage of shoots suitable for planting (Table 6). After 13 weeks from transplanting, the percentage of viable cv. Hedda shoots differed according to the recovery medium used for shoot tips after thawing ($p \leq 0.001$). The lowest viability in vivo was found on shoots that were placed on the medium M4 after thawing i.e. the one with GA_3 (Table 6).

Viability assessment of the blackcurrant core collection

After 9 weeks from thawing from cryopreservation, the recovery of shoot tips differed significantly between cultivars ($p \leq 0.001$). The average recovery was ca 90% for cvs. Åström, Karila and Brödtorp but less than 40% for cvs. Pyhtilän Musta, Ola and Hedda (Table 7). The recovery of shoot tips of cv. Melalahti was poor (only three recovered shoot tips out of 70 cryopreserved) and it was left out from the statistical tests. The duration of PVS2 treatment (25–30 min vs. 31–36 min) did not affect the recovery of shoot tips (p = 0.74). Within cultivars the mean variation in recovery after 9 weeks from thawing ranged 3–19% between cryopreserved sets and mean variation in recovery between thawed cryovials within cryopreserved sets ranged 4–23% (Table 7).

The mean recovery of shoot tips progressed differently among cultivars as culture in vitro proceeded ($p < 0.001$). Overall, the recovery of shoot tips was improved as the in vitro culture period was progressed from week 6 to week 9 but in cvs. Ola and Hedda the recovery results, on the contrary, decreased. The average recovery results of all the cultivars evaluated at weeks 6 and 9 were 48% and 63%, respectively ($p < 0.001$). The recovery was significantly better after nine weeks than after 6 weeks of cultivation in cvs. Brödtorp, Karila, Matkakoski, Nikkala, Osmolan Musta and Suvi-7 ($0.001 \leq p \leq 0.023$) as their mean recovery results were improved by 34%, 21%, 35%, 21%, 31% and 23%, respectively. In cv. Ola, the mean recovery result decreased by 18% from week 6 to week 9 (p = 0.017).

Table 6 The number of shoots of cv. Hedda transferred to in vivo cultivation from the recovery medium test and their estimated viability after 13 weeks in vivo

First recovery medium	Number of transplanted shoots size category		Viability of shoots %		
	1 cm	≥ 1.5 cm	Total	Mean	Cl
M2	39	21	60	87[a]	75–93
M3	17	43	60	95[a]	86–98
M4	28	8	36	61[b]	44–76

Cl 95% confidence intervals

Different superscript letters indicate significant differences ($p < 0.05$) between the recovery media used in the estimated viability of shoots in vivo.

Discussion

In the present study we focused on the development of modified droplet vitrification technique for cryopreservation of large diversity of blackcurrant cultivars representing the Finnish national core collection of blackcurrant.The results of our study demonstrate that cryopreservation by the modified droplet vitrification technique is a useful method for long-term ex situ conservation of blackcurrants, although there were big differences in recovery of shoot tips between the cultivars. In method testing the recovery of four cultivars ranged between 25.5–97.0%) (Table 2) and results of viability assessments in 22 cultivars ranged between 16.8%–93.8% (Table 7). However, cv. Melalahti showed no recovery after cryopreservation.

The plant collections in gene banks are aimed to secure the wide range of genetic and phenotypic variation of selected species. The variation between different traits of blackcurrant genotypes can be remarkable (Vagiri et al. 2016). In the study of Pluta et al. (2012) a high phenotypic diversity was confirmed among the blackcurrant genotypes from different geographic origins and those from similar geographic origins. Also, the study of Mattila et al. (2016) demonstrated a remarkable variation in contents of anthocyanins and flavonols among Finnish blackcurrants.

When large collections of accessions are cryopreserved, it can be a challenge to find cryopreservation protocol suitable for all of them (Reed et al. 2005). In the present study, the suitability of the cryopreservation method for the whole Finnish national blackcurrant collection was set as a goal. The droplet vitrification method was chosen to be optimized and used as a basic protocol for whole collection of blackcurrants, as it is reported to be a simple and universal protocol and easily implemented for wide range of accessions (Panis et al 2005; Romadanova et al. 2017). Moreover, as the protocol was in use in our laboratory with some modifications (Nukari and Uosukainen 2007) for cryopreservation of several other species, the method was easy to implement for laboratory infrastructure and facilities available.

The success of a cryopreservation protocol is affected by the combination of factors related to the characteristics of the source material and different steps of the protocol. The previous studies have shown that protocols may need to be adjusted, not only for selected species, but also for

Table 7 The estimated recovery rates for cryopreserved shoot tips of blackcurrant cultivars after 9 weeks from thawing. Replicates consisted of thawed cryovials in cryopreserved sets. The target number of thawed cryovials was three per each of three cryopreserved sets but the values varied due to a limited number of excised shoot tips in some cryopreserved sets. With some cultivars the cryopreservation was repeated more than three times in order to get 15 cryovials in storage. Variations in recovery rates between cryopreserved sets and thawed cryovials per sets are shown on the right

Cultivar	Cryopreserved sets	Vials thawed for recovery assessment		Recovery % of thawed cryovials		Variation in recovery % between cryopreserved sets		Variation in recovery % between vials within sets	
		per set[1]	in total	Mean	Cl	Mean	Cl	Mean	Std
Åström	3	3	9	93.8	81.4–98.2	3	0–16	6	6
Karila	3	3	9	91.1	75.9–97.1	7	0–24	9	5
Brödtorp	3	3	9	89.7	73.5–96.5	6	0–19	8	8
Osmolan Musta	3	3	9	86.7	68.2–95.2	6	0–23	12	10
Nikkala	3	3	9	80.8	59.2–92.5	7	0–21	4	3
Osmola	3	3	8*	75.6	51.2–90.2	19	4–43	23	15
Hietala	3	3	9	74.9	52.3–89.1	3	0–15	6	6
Venny**	3	3	9	73.3	50.3–88.2	19	5–41	18	5
Ri-289**	3	3	9	71.6	46.8 – 87.8	3	0–16	6	8
Vilma**	5	2–3	13	64.4	41.5–82.3	17	4–37	17	8
Kangosfors	3	3	9	61.1	35.7–81.6	18	5–39	19	3
Vertti**	4	2–3	10	60.1	33.3–82.0	17	5–35	18	11
Erkheikki	3	3	9	58.6	33.7–79.7	15	3–38	7	5
Gerby	3	3	9	51.9	27.9–75.0	13	2–32	17	18
Lepaan Musta	3	3	9	48.5	25.3–72.4	15	2–37	15	9
Suvi-7	3	3	8*	48.3	25.4–71.9	11	1–31	13	7
Matkakoski	3	2	6	47.9	23.8–73.1	5	0–17	8	8
Jänkisjärvi	3	2–3	7	45.7	22.5–71.0	7	0–22	14	19
Kuoksan Musta	3	3	9	41.7	20.3–66.7	5	0–17	7	7
Hedda	2	3	6	28.8	10.3–58.7	15	2–39	12	8
Ola	4	1–3	10	17.7	6.4 – 40.5	14	3–34	17	14
Pyhtilän Musta	3	1–3	7	16.8	5.9 – 39.2	19	4–45	19	6
Melalahti	3	2–3	7	–					

Cl confidence intervals

Std standard deviation

*Shoot tips from one cryovial was excluded due to contamination during in vitro culture

**Cultivars with green berries

different cultivars. Also, in the present study, the differences in post-cryopreservation recovery of shoot tips between cultivars were evident. Similarly, in the study of Benson et al. (1996), clear genotypic difference in shoot formation was found between blackcurrant cvs. Ben Tron (60%) and Ben More (20%) cryopreserved by vitrification procedure. However, Reed et al. (2005) found that the regrowth of the fifteen blackcurrant cultivars and accessions cryopreserved with the modified encapsulation-dehydration protocol ranged between 50–90% and was 80% and 70%, for cvs. Ben Tron and Ben More, respectively. The reason for those differences on recovery results may be related to the condition of source material, cryopreservation protocols used or differences of regrowth conditions.

The differences in the post-cryopreservation recovery of shoot tips between cultivars may also be related to their suitability for in vitro culture (Benson et al.1996). This might be likely especially with blackcurrants, as differences related to in vitro multiplication rates between blackcurrant cultivars and certain groupings of cultivars have been reported (Brennan et al. 1989). In the present study, the optimized protocol was tested with five cultivars selected due to their response to in vitro culture protocol used in our laboratory. In the preliminary assays (data not shown), cv. Hedda was characterized by leaf blackening during in vitro culture and production of only tiny rosettes without proper elongation of shoots. After cryopreservation, cv. Hedda also had blackening of leaves. Thus, the cv. Hedda with low suitability for in vitro culture also had low post-cryopreservation recovery. On the other hand, cv. Mortti, for instance, grew well both in vitro before cryopreservation, and in regeneration after thawing from cryopreservation.

In addition to cv. Hedda, also cv. Melalahti turned out to be recalcitrant to cultivation in vitro, which also affected the

post-cryopreservation success. The cv. Melalahti had low multiplication rates and severe browning of leaves during in vitro culture (data not shown) and no cryopreservation success. The cv. Hedda originates from a crossing of Öjebyn and Melalahti, (https://www.ribes-rubus.gf.vu.lt/download.htm). The partly common genetic background of cvs. Hedda and Melalahti may include recalcitrance both to in vitro cultivation and to their cryopreservation response. On the other hand, in vitro multiplication rate was also quite low for cvs. Matkakoski and Jänkisjärvi with no proper shoot elongation (data not shown). However their post-cryopreservation recovery was over 45%. The difficulties found in in vitro culture of the cvs. Matkakoski and Jänkisjärvi may be related to their very high latitude origin.

Our results show that modified droplet vitrifcation protocol was successful for most of the blackcurrant cultivars cryopreserved in the present study. To achieve desired cryopreservation success for all cultivars, re-optimization of the used cryoprotocol and in vitro culture practises especially for recalcitrant cultivars might be needed (Reed 2011). The methods for optimization of the cryoprotocol could include a longer cold acclimation period for in vitro cultures (e.g. Kushnarenko et al. 2009) or to treat shoot tips with antioxidants during pre-treatments or other steps of the protocol (e.g. Reed 2014, Mathew et al. 2019). To use PVS3 instead of PVS2 (Wang et al. 2019) or to alternation of recovery medium (Chang and Reed 1999) may help to improve the cryoprotocol.

The results of cvs. Hedda and Mikael showed that the importance of the first recovery medium used after thawing and the effect of the medium components on recovery of shoot tips may vary between cultivars. The cv. Hedda had quite poor post-cryopreservation recovery in the first viability assessment (Table 2), but improved recovery was achieved when GA_3 was left out from the first recovery medium used after thawing (Table 3). In cv. Mikael, the first recovery medium had no influence on the shoot recovery rate (Table 3), but it affected blackening and hyperhydricity of the shoots (Table 4). Based on the results on recovery medium, it seemed that the presence of GA_3 may have negative effect on recovery of some blackcurrant cultivars. Thus, the medium M3 was chosen as the first recovery medium to be used for blackcurrants further on. Previously, the negative effect of GA_3 on the regrowth of some apple genotypes has been reported (Condello et al. 2011). However, in the case of cv. Hedda, beneficial effect of the M3 medium on recovery of shoot tips was neither detected in later viability assessments.

The efficiency of the cryopreservation process may be improved with several ways (Volk et al. 2014). In the present study, we examined how many weeks thawed shoot tips of blackcurrant should be cultured in vitro to get a reliable result of their recovery. According to the results of four cultivars used to test the suitability of the protocol, there was no need to prolong in vitro culture up to 12 weeks unless shoots were meant to be transferred in vivo. The results from the viability assessments of 22 blackcurrant cultivars were used to evaluate if the regeneration time of the thawed shoot tips could be shortened from 9 to 6 weeks. Our results indicated that after 6 weeks of in vitro culture, the recovery result of the tested blackcurrant cultivars can be expected to remain or improve with most of the cultivars. However, the results evaluated after 6 weeks of in vitro culture gave too optimistic view related to the regeneration for some cultivars. Thus, in the present study, the assessment of viability 9 weeks after thawing seems to be a preferable practise over the assessment after 6 weeks.

When accessions are cryopreserved for long-term preservation, the predicted viability level of each preserved sample set is estimated based on the recovery of thawed control samples of the same set. The number of vials stored, and the number of vials thawed usually vary according to the cryopreserved species and their later use. Therefore, no precise instructions for number of vials or samples to be cryopreserved are available, but the guidelines for determination of the number of thawed control vials and stored vials are given by Dussert et al (2003). Later Volk et al. (2017) have presented series of tools and tables to aid genebank managers to design cryobanking strategies.

According to the Decision tree by Volk et al. (2017), one or two thawed control vials of ten explants might be enough to assess the number of explants predicted to be viable, based on the total number of stored explants and the viability level. In the present study, the number of cryovials used for viability assessments was in most cases three per each cryopreservation set. In the method testing phase, each cultivar was cryopreserved in four sets. Thus, altogether 12 vials per cultivar was thawed and in addition 12 vials were stored for long-term preservation. Later, during the routine conservation work, the practise was in most cases to repeat cryopreservation three times per cultivar, and in each cryopreservation occasion to store five cryovials in cryotank and to use three vials for viability assessments. In our study, the variation in recovery percentage between cryopreserved sets and variation in recovery percentage between thawed cryovials per set was tested. In both cases, the mean variation was quite moderate (Table 7). Thus, the thawing of two cryovials per each set for viability assessment might probably have been enough for most of the cultures cryopreserved in the present study. However, at least one control vial needs to be thawed per each cryopreserved set to reveal the possible contaminations that might have happened during the cryopreservation process.

In the present study, the microshoots of four blackcurrant cultivars transplanted in vivo after cryopreservation were rather short. Also the proportion of the tallest

shoots was quite low (Table 2). Regarding to the transfer of microshoots from in vitro to in vivo, the shoots one centimeter tall seemed to be adequate for transplanting, but the shoots taller than those were more vigorous, and they should, therefore, be prioritized if possible. Shoot elongation might be gained by adjusting the growth regulators used in tissue culture medium before the transfer of shoots to in vivo. The number of shoots adequate for transplanting in vivo can also be increased by a longer in vitro culture time. This should be taken into consideration when planning the regeneration schedules of material from cryopreservation.

Based on the calculations of Volk et al. (2017), for 100 explants cryopreserved with 40% viability level, the number of explants predicted to be viable is 32 (with the confidence level of 95%). In the present study, the overall number of shoot tips derived from each cultivar and intended for long-term cryopreservation was at least 100. The 40% regrowth rate of in vitro controls was set as a goal for cryopreservation to be regarded as successful. This was based on estimations of average survival and regrowth and on the average number of stored cryovials, which in most cases was 15. Altogether 23 cultivars out of 27 had an average recovery rate above 40%. Based on the calculations of Volk et al. (2017), for cultivar Pyhtilän Musta, which held the lowest viability level, only 16.8%, the number of shoot tips predicted to be viable out of the 150 shoot tips set to cryopreservation, was 16 (with the confidence level of 95%).

We conclude that cryopreservation by the optimized droplet vitrification method is suitable for long-term preservation of *R. nigrum* collections. It was shown, that after cryopreservation shoot tips could be recovered via in vitro culture and shoots could be multiplied for in vivo cultivation. However, to improve recovery result, some optimization of in vitro practices should be considered for cultivars being recalcitrant to in vitro cultivation. This might contribute to recovery after cryopreservation and likely shorter micropropagation time needed to get shoots tall enough for successful transplanting in vivo. The cryopreserved shoot tips of blackcurrant cultivars in cryobank can offer a valuable source for future, considering the potential needs for specific cultivars in changing environmental conditions.

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Author contributions SR and AN designed the research. SR performed the optimization of cryoprotocol, testing of the selected protocol and wrote the paper. AN and ST took part in the long-term cryopreservation of cultivars. JK was responsible for the statistical analyses. JL was responsible for the plant health testing of the stock plants, JK, AN, JL, ST, SK, MV, and HH took part in the writing process. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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