



Establishment of an efficient and wide-spectrum droplet-vitrification cryopreservation for raspberry (*Rubus idaeus* L.) germplasm and assessments of genetic integrity and vegetative growth in the regenerants

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Abstract

By optimizing size of shoot tips, preculture medium and exposure duration to PVS2, we established an efficient and wide-spectrum droplet-vitrification cryopreservation for shoot tips of raspberry (*Rubus idaeus* L.). This protocol yielded 80–100% and 67–100% of survival and shoot regrowth levels in cryopreserved shoot tips across 23 raspberry genotypes. Genetic integrity was assessed in cryo-derived regenerants after 3 months of post-cryopreservation using inter simple sequence repeat (ISSR), single nucleotide polymorphism (SNPs), and insertions and deletions (InDels). ISSR did not detect any polymorphic bands in the cryo-derived regenerants. Although the number of SNPs and InDels decreased in the cryo-derived regenerants, variation trends were similar between the cryo-derived regenerants and the control. Plant vegetative growth and root growth were assessed in the cryo-derived plants after 9 weeks of growth in greenhouse. There were no significant differences in plant vegetative growth measured by plant height, number of fully-opened leaves, leaf area, and fresh and dry weight between the cryo-derived plants and the control, although significant differences were observed in root growth measured by root total length, root average diameter and root volume between the two types of plants. The results obtained in the present study indicate that the droplet-vitrification method has great potential for cryopreservation of raspberry germplasm.

Key message

Establishment of a droplet-vitrification cryopreservation of *Rubus* shoot tips provided technical supports for cryopreservation of diverse genetic resources of raspberry.

Keywords Cryopreservation · Field performance · Inter simple sequence repeat · Raspberry · Single nucleotide polymorphism

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Introduction

Raspberry (*Rubus idaeus* L.), one of the most economically important berry crops (De Ancos et al. 2000), healthy and appreciated by consumers, and widely grown in Asia, eastern and southern Africa, Europe and North America (Jennings et al. 1991). The world gross production index number of raspberry has increased 50.87 from 2012 to 2022 (FAO. 2023). Availability of and easy access to diverse genetic resources are prerequisite of breeding of elite cultivars in both the traditional and biotechnological strategies.

Cryopreservation is currently considered the most reliable strategy for long-term and safe conservation, both preserving the genetic variation or gene resources, but also for preserving the plant health status of virus-free nuclear stock materials (Panis 2019; Bettoni et al. 2021; Wang et al. 2021a; Popova et al. 2023). Over the past few decades, several methods have been described for raspberry cryopreservation, including slow-freezing (Sakai and Nishiyama 1978), encapsulation–vitrification (Wang et al. 2005), encapsulation–dehydration (Wang et al. 2005; Kovalchuk et al. 2010), PVS2 vitrification (Kovalchuk et al. 2010) and droplet-vitrification (Condello et al. 2011; Ukhatoeva et al. 2017). Nevertheless, availability of efficient and wide-spectrum cryopreservation methods is necessary for cryobanking diverse germplasm of raspberry.

Currently, droplet-vitrification cryopreservation is the most widely applicable cryo-protocol for preserving plant germplasm within gene banks (Panis et al. 2005; Sakai et al. 2008; Wang et al. 2018, 2021b; Panis 2019; Normah et al.

2019; Gowthami et al. 2023; Malhotra et al. 2024). Existing studies showed shoot regrowth rates in cryopreserved shoot tips of red raspberry by droplet-vitrification were still low and unstable: 18% in ‘Latham’ (Condello et al. 2011) and 24–89% in 12 genotypes (Ukhatoeva et al. 2017). Therefore, further optimization of major parameters involved in droplet-vitrification for raspberry shoot tips is still needed.

In vitro culture and the major steps of cryopreservation, such as preculture and exposure to plant vitrification solution (PVS) all impose stress to the samples subjected to cryopreservation and may induce genetic and morpho-genetic variations in cryo-derived regenerants. Therefore, assessments of genetic integrity and field performance in the cryo-derived regenerants are necessary (Harding 2004; Wang et al. 2021b). Molecular markers such as inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), and single sequence repeats (SSR) have been frequently used for assessments of genetic integrity in the cryo-derived regenerants (Feng et al. 2013; Yin et al. 2014; Zhang et al. 2015; Li et al. 2017; Wang et al. 2021b). Results of assessments of genetic integrity in the cryo-derived regenerants obtained so far were generally promising (Wang et al. 2021c). Assessments of field performance were conducted mainly by measurements of survival (Agrawal et al. 2014), root formation and growth (Zhang et al. 2015; Zhang et al. 2020), vegetative and reproductive growth (Bi et al. 2016), and tuber production (Li et al. 2017) in cryo-derived plants grown in greenhouse. Results showed the cryopreservation did not have significantly negative effects on cryo-derived plants, and even increased growth of them (Wang et al. 2021c).

Studies on assessments of genetic integrity and field performance of cryo-derived regenerants of raspberry have been quite limited. Using ISSR, Ukhatoeva et al. (2017) reported genetic integrity was maintained in the cryo-derived regenerants of red raspberry. The present study was, therefore, to establish an efficient and wide-spectrum droplet-vitrification method for cryopreserving shoot tips of diverse raspberry germplasm, and assess genetic integrity and field performance in cryo-derived regenerants.

Materials and methods

Plant material

Raspberry ‘Stiora’ (Fig. 1a) was used to optimize droplet-vitrification protocol for cryopreservation and to assess genetic stability and field performance in cryo-derived regenerants. Twenty-two additional raspberry genotypes were subsequently used for testing the optimized cryopreservation protocol (Table 1). In vitro stock shoots of all

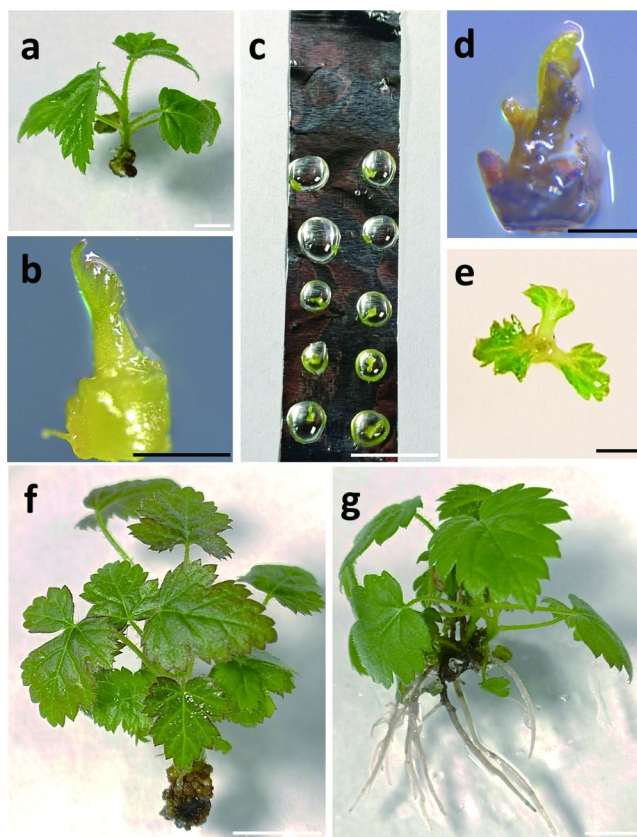


Fig. 1 Droplet-vitrification cryopreservation of shoot tips of Raspberry ‘Stiora’. In vitro stock shoot from which shoot tips were excised for cryopreservation (a). A shoot tip used for cryopreservation (b). Droplets on aluminium foil (c). A cryo-derived regenerant after 1 week of post-culture (d). A cryo-derived regenerants after 4 weeks of post-culture (e). A cryo-derived regenerant after 3 months of post-culture (f). An in vitro cryo-derived rooted plantlet (g). Which scale bars = 1 cm, black scale bars = 1 mm

Table 1 Droplet-vitrification cryopreservation of raspberry genotypes. Ten shoot tips have been cryopreserved in each cryotube in the liquid nitrogen

Genotypes	Number of cryotubes	Survival rate (%)	Regrowth rate (%)
Agat	15	96	95
Anitra	17	96	96
Asker	15	94	48
Balder	10	97	97
Borgund	12	100	98
Cascade Delight	12	85	69
Dino	20	100	95
Glen Ampel	18	99	96
Glen Fyne	15	98	92
Ninni	15	100	95
Norfolk Giant	8	96	93
Orion	12	98	92
Preussen	14	96	86
<i>R. occidentalis</i>	8	91	88
RU004 03067	12	97	95
Duo	17	99	95
Tulamen	11	100	92
Varnes	12	100	91
Vene	12	100	80
Veten	17	93	88
Stiora	15	96	96
Fair View*	3	100	96
Zlatá Královna*	6	97	86

* These cultivars have only been tested for cryopreservation but have not been stored in the cryotank in Norway.

raspberry genotypes used in the present study were cultured on shoot maintenance medium (SMM) containing 4.4 g/L Murashige and Skoog (1962; MS), 30 g/L sucrose, 0.5 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L indole-3-butyric acid (IBA), 36 mg/L ethylenediamine di-2-hydroxyphenyl acetate ferric (Fe-EDDHA), and 6 g/L agar. The pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min. The cultures were maintained at 22 ± 2 °C under a 16-h photoperiod with a light intensity of $50 \mu\text{mol s}^{-1} \text{m}^{-2}$ (provided by cool-white fluorescent tubes). Subculturing was performed every 4 weeks.

Optimization of droplet-vitrification protocol

Size of shoot tips Three different sizes of Raspberry ‘Stiora’ shoot tips were used for optimizing droplet-vitrification protocol. They are 0.5 mm in length with 1–2 leaf primordia (LPs), 1 mm in length with 3–4 LPs (Fig. 1b) and 2 mm in length with 5–6 LPs. Shoot tips were excised from 4-weeks old stock shoots (Fig. 1a) and step-wise precultured with 0.25 M sucrose for 1 day, 0.5 M sucrose for 1 day, and 0.75 M sucrose for 1 day. The shoot tips were then transferred to a

loading solution containing 2 M glycerol and 0.4 M sucrose for 20 min at room temperature (RT). After loading, the shoot tips were exposed to plant vitrification solution 2 (PVS2, Sakai et al. 1960) at RT for 20 min. PVS2 was composed of 4.4 g/L MS, 15% (w/v) ethylene glycol, 15% (w/v) dimethylsulfoxide (DMSO), 30% (w/v) glycerol and 0.4 M sucrose (pH 5.8). Following vitrification, the shoot tips were transferred into PVS2 droplets (15 μl for each) on aluminium foil (about 3×0.8 cm, Fig. 1c) and directly immersed in LN. After LN treatment for 2 min (for establishment of cryopreservation protocol, 1 h was used for cryopreservation of genotypes), aluminium foil strips were removed out from LN and rapidly transferred into an unloading solution for 20 min at RT. Unloading solution contained 0.75 M sucrose in MS medium. The cryopreserved shoot tips were post-cultured on 0.75 M sucrose medium in the dark for 3 days, and then transferred on SMM medium in the light condition (22 ± 2 °C, 16-h photoperiod, $50 \mu\text{mol s}^{-1} \text{m}^{-2}$ provided by cool-white fluorescent light) for recovery. Subculturing performed every 4 weeks.

Preculture Shoot tips (Fig. 1b), measuring approximately 1 mm, were subjected to different sucrose concentrations (0.25 M, 0.5 M, and 0.75 M) for different durations for 3 days. A stepped-wedge design was employed to assign combinations of sucrose concentration and preculture duration, with treatments labeled A to G (Table S1). For instance, in treatment D, shoot tips were precultured sequentially for one day each at 0.25 M, 0.5 M, and 0.75 M sucrose concentrations. All subsequent procedures, including loading, vitrification, unloading, and post-culture, followed the same protocol as previously described for optimizing shoot tip size.

PVS2 vitrification Shoot tips (1 mm in length, Fig. 1b) were exposed to PVS2 at RT for varying durations of 0, 5, 10, 20, 40, and 60 min to determine the optimal vitrification time. Other procedures including preculture, loading, unloading, and post-culture were the same as described above for optimizing size of shoot tips.

Post-culture Following cryopreservation, the shoot tips were post-cultured on 0.75 M sucrose medium in darkness for durations of 0, 1, 3, and 7 days, respectively, and then transferred to the light condition for recovery. Other procedures including preculture, loading, vitrification, and unloading were the same as described above for optimizing size of shoot tips.

Testing the optimized droplet-vitrification protocol to different raspberry genotypes To evaluate the broad-spectrum applicability of the optimized droplet-vitrification protocol

developed in this study, 22 raspberry genotypes (Table 1) from an in vitro collection in Norway were tested. For each genotype, 8 to 20 cryotubes were used, with 10 shoot tips cryopreserved in liquid nitrogen (LN) per cryotube. Additionally, 4 to 5 shoot tips were used as controls to assess survival and regeneration rates. Notably, the cultivars Raspberry ‘Fair View’ and ‘Zlatá Královna’ were tested with 30 and 60 shoot tips, respectively, for cryopreservation; however, they were not stored in the cryotank in Norway.

Assessments of genetic integrity in cryo-derived regenerants

After three months of post-culture, cryo-derived regenerants (Fig. 1f) of Raspberry ‘Stiora’ were collected and used for assessments of genetic integrity by ISSR, SNPs and InDels. In vitro cultured shoots were used as control.

Total DNA was extracted using the DNeasy plant Mini Kit (Qiagen GmbH, Hilden, Germany) with minor modifications (home-made lysis buffer have been applied to replace buffer AP1 in kit), following the manufacturer’s instructions. The lysis buffer contains 6 mM Tris-HCl (pH 8.0), 12 mM EDTA (pH 8.0), 17.4 g NaCl, 6 g Sodium dodecyl sulfate (SDS), and water to a total volume of 100 ml.

ISSR was performed as described by Zhang et al. (2015). Ten primers were selected from 30 candidates based on the number of amplifications. Each polymerase chain reaction (PCR) reaction contained 2.5 µL of 10 × PCR buffer, 0.75 µL of MgCl₂ (50 mM), 0.5 µL of dNTP Mix (10 mM), 0.5 µL of primer (10 µM), 1.25 µL KB Extender, 0.1 µL Platinum™ *Taq* DNA Polymerase (Invitrogen, USA), and 1 µL of template DNA (50 ng/µL). PCR conditions included an initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 30 °C for 45 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. Each resulting PCR product (10 µL) was subjected to electrophoresis in a 1.2% agarose gel previously stained with SYBR safe DNA stain (Invitrogen, ThermoFisher Scientific, USA), and their sizes were estimated using a 100 bp DNA ladder (New England BioLabs Inc, Ipswich, UK).

Paired-end sequencing was conducted on the DNBseq platforms (BGI TECH SOLUTIONS (HONGKONG) CO., LIMITED) with an average depth of 15-fold genome coverage. Cleaned reads were mapped against the reference genome of Raspberry ‘Anitra’ (Davik et al. 2022). The MarkDuplicates (Picard, <https://sourceforge.net/projects/Picard/>, version 2.25.2) was then applied to remove the potential PCR duplicates. GATK software v4.1.0.0 (McKenna et al. 2010) was used to perform SNP (single nucleotide polymorphisms)

calling. Raw vcf files were filtered using the GATK standard filter method with the following parameters: “QD < 2.0 || mq < 40.0 || FS > 60.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0” for SNP and “QD < 2.0 || FS > 200.0 || ReadPosRankSum < -20.0” for InDels. To assess the SNP rates in cryopreserved and in vitro cultures compared to the Raspberry ‘Anitra’ genome, a sliding-window approach (2 m windows sliding in 1 m steps) was applied.

Assessments of field performance in cryo-derived plants grown in greenhouse

Cryo-derived and in vitro (control) shoots (> 2 cm in length) of Raspberry ‘Stiora’ were rooted (Fig. 1g) and grown in greenhouse at 24 ± 2 °C with a 16-h photoperiod of light intensity of 200 µmol·m⁻²·s⁻¹. Root and shoot growth were measured after 9 weeks of growth. Shoot growth was measured in plant height, number of fully opened leaves, leaf area, fresh weight, and dry weight. Leaf area was measured from the top three fully opened leaves, using WinFOLIA™ (EPSON, WinRHIZO Pro2003a, Regent Instruments Inc., Quebec, Canada). Root growth was measured in root total length, root average diameter, root surface area, and root volume by WinRHIZO™ (EPSON 1680, WinRHIZO 2003d, Regent Instruments Inc., Quebec, Canada).

Experimental design and data statistical analysis

In all experiments, the optimal shoot tip size, preculture, PVS2 treatment time, and post culture were determined. Each treatment includes ten shoot tips, and three replicates were conducted in two independent experiments. The optimized droplet-vitrification protocol was assessed across different raspberry genotypes with thirty shoot tips of each genotype utilized. Survival and regrowth rates were defined as the ratio between survival and regrowth numbers/total numbers of treatment shoot tips, and recorded after 7 days and 4 weeks of post-culture.

Genetic stability was evaluated using ISSR analysis on twenty cryopreserved samples and ten in vitro cultures (control) derived from in vitro plants. In addition, InDels and SNPs analysis were performed on five cryopreserved plants and five in vitro cultured (control) samples, with three replicates for each experiment. Furthermore, field performance was evaluated by comparing twenty cryopreserved plants with twenty in vitro (control) plants in greenhouse setting. Statistical analysis of significant differences among treatments was performed using Student’s *t*-test (*P* < 0.05).

Results

Effect of droplet vitrification protocol on survival and regrowth

Surviving shoot tips began exhibiting green colour after 4 days of post-culture following cryopreservation (Fig. 1d). Shoot tips regrowth, defined by the emergence of three fully open leaves, was observed after 4 weeks (Fig. 1e). In the optimization experiment of shoot tip size (Fig. 2a), shoot tips measuring 1 mm showed significantly higher survival (100%) and regrowth (97%) than those with sizes of 0.5 mm and 2 mm (Fig. 2b). In the preculture experiment, the concentration of sucrose significantly affected the regrowth. The shoot survival rates, all above 80%, did not show significant variation across treatments. However, the shoot regrowth rates were significantly higher in treatments B (0.25 M sucrose for 2 days and followed by 0.5 M sucrose for 1 day, 74.5%), C (0.25 M sucrose for 1 day and followed

by 0.5 M sucrose for 2 days, 70%), and D (one day each at 0.25 M, 0.5 M, and 0.75 M sucrose concentrations, 75.8%) compared to treatments A (32.1%), E (34.6%), F (42.9%), and G (24.4%) (Fig. 2c). During the optimization of vitrification durations, both shoot survival and regrowth increased as the exposure time to PVS2 solution was extended from 0 to 20 min, then plateaued or declined slightly toward 60 min. The highest survival rate (100%) was observed at 20 min of PVS2 treatment, with a corresponding regrowth rate of 94% (Fig. 2d). In the post-culture experiment, post-culturing with a sucrose concentration of 0.75 M significantly affected shoot recovery. The shoot survival rates did not significant changes between control (0 day) and treatment (1, 3, and 7 day). However, the shoot regrowth of treatment groups was significantly higher than control group, the highest survival rate (87.6%) was obtained when shoot tips were post-cultured onto a 0.75 M sucrose medium for 1 day (Fig. 2e).

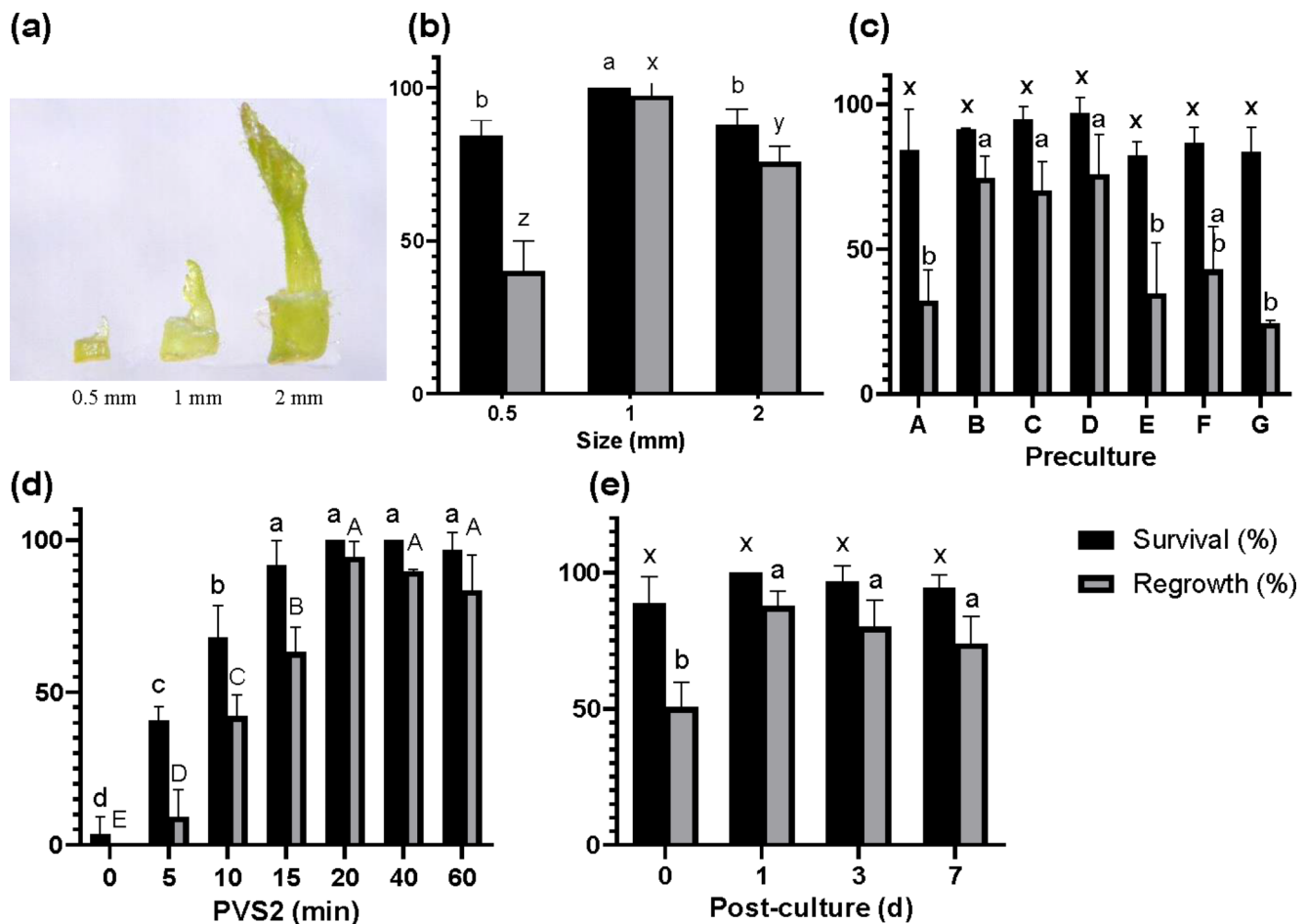


Fig. 2 Shoot tip size, preculture, PVS2 durations, and post-culture effects on survival and regrowth of cryopreservation of Raspberry 'Stiora'. Shoot tip size (**a**). The effects of shoot tip size on survival and regrowth (**b**). The effects of preculture with different sucrose concentrations on survival and regrowth (**c**). The effects of PVS2 time on

survival and regrowth (**d**). The effects of durations on 0.75 M sucrose post-culture medium on survival and regrowth (**e**). Data are presented as mean $\pm$ SE and analyzed using Student's *t* test. Least significant differences were calculated at $P < 0.05$

Table 2 ISSR primer names, sequences, and number of amplified bands in in vitro plants from in vitro culture (control) and cryopreserved shoot tips of raspberry ‘Stiora’

Primer name	Primer sequence (5'-3')	Annealing temperature (°C)	Number of monomorphic bands / primers	Number of polymorphic bands / primers
D2	(GA) ₉ C	30	6	0
3 A 37	(ca.) ₇ TGA	30	11	0
UBC857	(AC) ₈ YG	30	10	0
UBC811	(GA) ₈ C	30	5	0
UBC834	(AG) ₈ YT	30	6	0
UBC825	(AC) ₈ T	30	7	0
UBC835	(AG) ₈ YC	30	10	0
UBC866	(CTC) ₆	30	6	0
UBC868	(GAA) ₆	30	4	0
UBC889	DBD*(AC) ₇	30	5	0

Testing of genotype response to droplet vitrification

When the optimized droplet-vitrification method was tested to other 22 raspberry genotypes, high survival rates ranged from 85–98% across most genotypes. Regrowth rates varied, particularly in genotypes like ‘Asker’ (48%) and ‘Cascade Delight’ (69%) displaying lower regrowth, while others like ‘Borgund’, ‘Dino’, and ‘Ninni’ showed regrowth rates of 95% or higher. The results suggest a generally effective cryopreservation protocol (Table 1).

Genetic stability assessment after cryopreservation

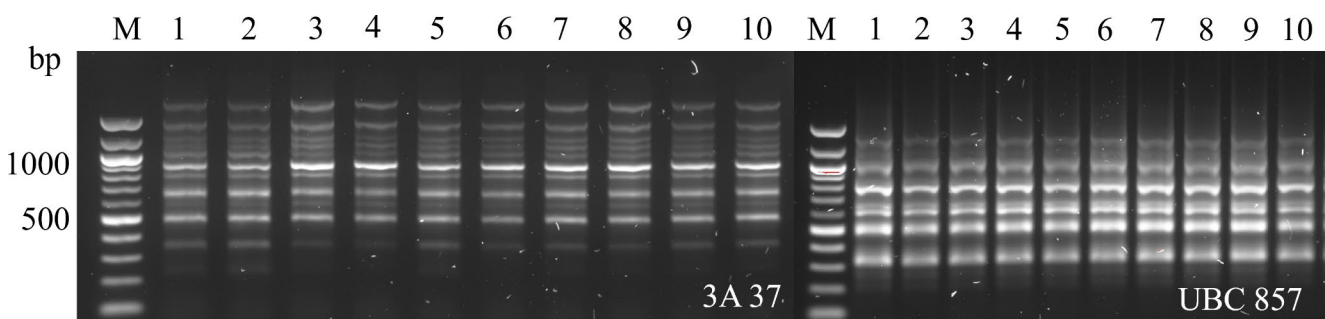
Out of the 30 primers tested in the ISSR analysis, 10 primers were selected with clear and strong bands (Table 2). Across all tested plants, a consistent count of 70 clear bands in

Table 4 Comparison of genetic variation levels between cryopreserved shoot tips (+LN) and in vitro cultures (-LN) compared to the reference genome of raspberry ‘Anitra’

Types of plant	Number of InDels	Number of SNPs	Mean of SNP rate (%)
-LN	22,844	233,031	0.942
+LN	22,216	229,722	0.928

total were detected using these selected primers. The number of bands produced by each primer ranged from 4 to 11 (Table 2). In total, 1400 bands were recorded across the 20 cryo-derived plants analyzed. No polymorphic bands were detected in any of the plants regenerated from cryopreserved shoot tips compared to the in vitro control (Table 2; Fig. 3).

Shoot samples from in vitro culture and cryopreserved shoots were subjected to sequencing, resulting in a total of 10.88 Gb of clean bases with an average coverage depth of 14.38× of the reference genome raspberry ‘Anitra’ across both samples. Approximately 92% of these reads were accurately mapped to the reference genome, indicating high data quality (Table 3). For precise variant identification, single-nucleotide polymorphism (SNP)/InDel variants were filtered through a series of steps following detection by GATK. In total, 229,722 SNPs and 22,216 InDels were identified from in vitro culture shoots, while cryopreserved shoots exhibited a lower number of SNPs (229,722) and InDels (22,216) with a reduced SNP rate of 0.928% (Table 4). Analysis of the SNP rate distribution across chromosomes showed a generally uniform pattern, with cryopreserved shoots displaying similar trends of variation to those observed in shoots from in vitro culture (Fig. 4).

**Fig. 3** ISSR banding pattern in plants regenerated from in vitro culture (control) and cryopreserved shoot tips of Raspberry ‘Stiora’. M marker, lanes 1–5 plants regenerated from in vitro cultures, lanes 6–10 plants regenerated from cryopreserved shoot tips**Table 3** Summary of DNA sequencing and comparison of genetic variation levels between cryopreserved shoot tips (+LN) and in vitro cultures (-LN)

Types of plant	Number of clean reads (M)	Number of clean base (Gb)	Ratio of Q30 base (%)	Ratio of GC (%)	Sequencing depth (×)	Mapped ratio (%)
-LN	18.12	5.44	91.65	38.39	14.8	91.91
+LN	18.14	5.44	91.82	38.38	13.96	91.54

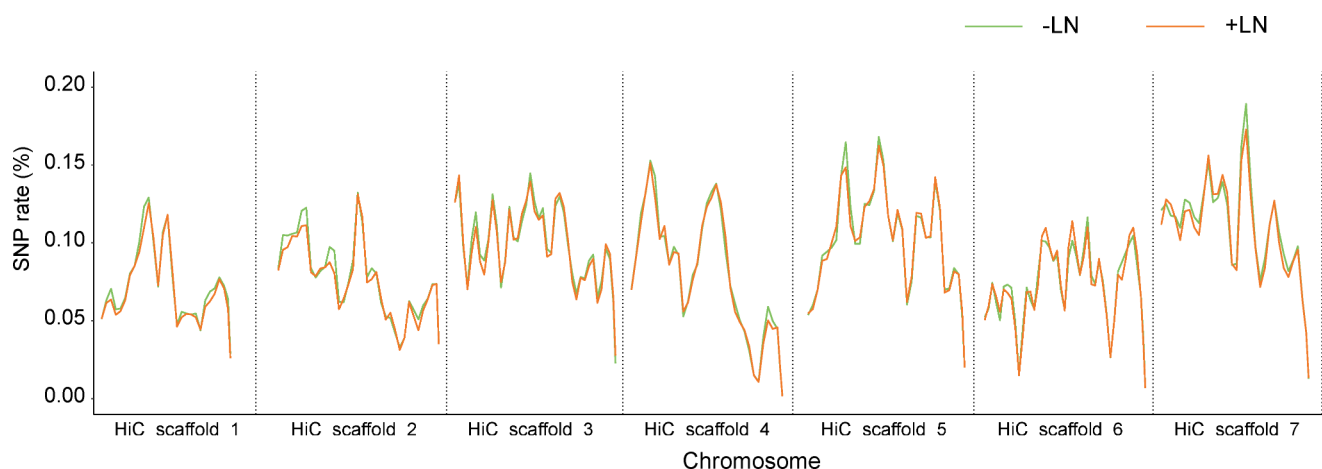


Fig. 4 The SNP rates across the Chromosome in cryopreserved shoot tips (+LN) and in vitro cultures (-LN) compared to reference genome Raspberry ‘Anitra’

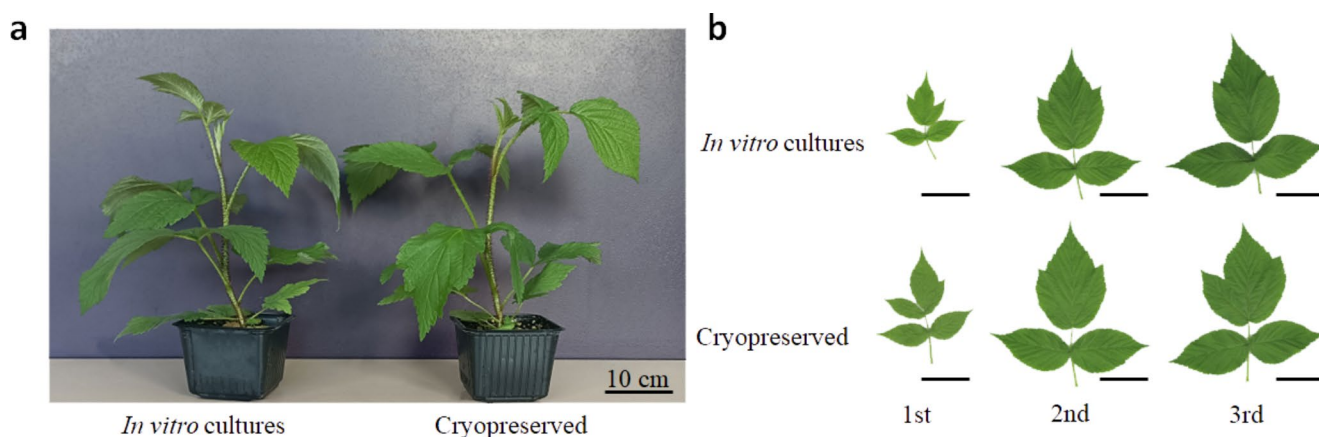


Fig. 5 Field performance of plants regenerated from cryopreserved shoot tips and in vitro cultures of Raspberry ‘Stiora’. Vegetative growth in plants regenerated from cryopreserved shoot tips and in vitro cultures after 4 weeks of growth under greenhouse conditions (a).

Morphologies of leaves of plant regenerated from cryopreserved shoot tips and in vitro cultures after 9 weeks of vegetative growth under greenhouse condition (b). Scale bars without number = 5 cm

Table 5 Comparison of vegetative growth in plants regenerated from cryopreserved shoot tips (+LN) and in vitro cultures (-LN) of Raspberry ‘Stiora’

Types of plant	Plant height (cm)	Fully opened leaves	Fresh weight (g)	Dry weight (g)	Leaf area (cm ²)
-LN	49.7 ± 4.4 a	9.4 ± 0.6 a	49.4 ± 3.63 a	10.3 ± 0.98 a	153.2 ± 14.9 a
+LN	51.7 ± 3.1 a	10.1 ± 0.5 a	51.3 ± 2.70 a	10.7 ± 0.63 a	153.9 ± 16.4 a

Results were recorded after 9 weeks of vegetative growth. Data were presented as means ± SE, and with different letters in the same column indicate significant differences at $P < 0.05$ by the Student’s t test ($n = 30$).

Field performance in greenhouse-grown plants

After 4 weeks of growth, plants regenerated from cryopreserved shoot tips exhibited similar growth patterns to control plants (Fig. 5a). After 9 weeks of growth, vegetative growth parameters were compared between plant regenerated from cryopreserved shoot tips and those from in vitro

cultures. Measures such as plant height, number of fully opened leaves, fresh weight, and dry weight showed no significant differences between the two groups (Table 5). The morphologies of leaves were identical between cryopreserved and in vitro cultured plants (Fig. 5b), and there was no significantly difference observed in the leaf area of fully opened leaves (Table 5).

Table 6 Comparison of root formation in plants regenerated from cryopreserved shoot tips (+LN) and in vitro cultures (-LN) of Raspberry ‘Stiora’

Types of plant	Root total length (cm)	Root AvgDiam (mm)	Root SurfArea (cm ²)	Root Volume (cm ³)
-LN	5652.0 ± 658.0 b	0.57 ± 0.02 a	1001.6 ± 112.2 a	14.2 ± 1.7 a
+LN	7243.1 ± 601.7 a	0.43 ± 0.02 b	966.3 ± 71.2 a	10.3 ± 0.8 b

Results were recorded after 9 week of root formation. Data were presented as means ± SE and with different letters in the same column indicate significant differences at $P < 0.05$ by the Student's t test ($n = 30$). AvgDiam = Average Diameter, SurfArea = Surface Area

Root parameters were assessed after 9 weeks of growth in the greenhouse. Significant differences were noted between cryopreserved and in vitro cultured plants. While cryopreserved plants exhibited longer roots (7243.1 cm) compared to those from in vitro culture (5652.0 cm), the average diameter and volume of roots were lower in cryopreserved plants (0.43 mm and 10.3 cm³) than in plants from in vitro culture (0.57 mm and 14.2 cm³) (Table 6). However, no significant difference was observed in root surface area between cryopreserved shoot tips (966.3 cm²) and those from in vitro culture (1001.6 cm²; Table 6).

Discussion

In this study, we optimized the droplet-vitrification protocol using Raspberry ‘Stiora’. Subsequently, shoot tips from 22 raspberry genotypes were successfully cryopreserved, with shoot regrowth rates ranging from 48 to 98%. Field performance and genetic stability of Raspberry ‘Stiora’ plants derived from cryopreserved shoot tips were compared to those from in vitro cultures.

In droplet vitrification, the four key factors influencing shoot regrowth of cryopreserved shoot tips are shoot tip size, preculture, vitrification solution (PVS2), and post-culture (Kim et al. 2006; Yin et al. 2014; Liu et al. 2017). In our study, we have optimized all these parameters. The results identified an optimized cryopreservation protocol for 1 mm raspberry shoot tips with high survival and regrowth rates. The shoot tips were first precultured in a stepwise sucrose medium: 0.25 M sucrose for 1 day, 0.5 M sucrose for 1 day, and then 0.75 M sucrose for 1 day. Afterwards, the shoot tips were exposed to PVS2 for 20 min at room temperature before rapidly immersing in liquid nitrogen. Following storage, the shoot tips were recovered in a 0.75 M sucrose medium for 3 days. To our knowledge, this represents the most efficient and wide-spectrum droplet-vitrification cryopreservation method for raspberry (*Rubus idaeus* L.) germplasm (Sakai and Nishiyama 1978; Wang et al. 2005;

Kovalchuk et al. 2010; Condello et al. 2011; Ukhatova et al. 2017).

Preserving biological materials in LN ensures that all cell division and physiological metabolism within the tissues remain in a state of dynamic stagnation, keeping them alive. As a result, shoot tips do not develop genetic mutations during cryopreservation (Wang et al. 2021a). However, in addition to liquid nitrogen step, various preculture, PVS2, and post culture treatments before and after cryopreservation may induce genetic alterations in the preserved materials (Benson 2008; Harding 2004; Wang et al. 2014).

For genetic stability, molecular markers such as ISSR and amplified fragment length polymorphism (AFLP) have been widely used to assess genetic stability after cryopreservation (Harding 2004; Wang et al. 2014). Merhy et al. (2014) used ISSR analysis to determine that the shoots derived from explants were genetically stable in the genomic regions. Li et al. (2014) used ISSR analysis on cryopreserved *Malus* shoot tips and did not find any polymorphic bands. Zhang et al. (2020) used ISSR and AFLP analysis on cryo-derived *Actinidia spp.* shoots and did not detect any polymorphic bands. In our investigation, we evaluated genetic stability using ISSR and observed no polymorphic bands in either cryo-derived shoots or in vitro culture shoots (Fig. 3). However, it is important to note that these techniques only study a small portion of the genome, which may not fully reflect the true extent of genetic variation. Wang et al. (2022) recently reported the use of next-generation sequencing (NGS) to compare four sweet orange callus samples after 30 years of constant tissue culture with newly induced callus, providing new insights into assessing genetic variation. In our present study, we performed SNPs and InDels analysis using NGS, by sequencing the entire Raspberry ‘Stiora’ genome. The variation trend of SNP rate on chromosomes was similar between cryopreserved shoots and in vitro culture (Fig. 4). However, the cryo-derived shoots exhibited a lower detectable SNP rate (Table 4). Zhou et al. (2023) reported that DNA methylation changes in the early-ripening bud sport of peach resulted in a large-scale behavior of allelic dropout and imbalance. This is similar to our research findings, where the lower SNP count detected in frozen-derived branches compared to in vitro cultures may be attributed to increased sensitivity of frozen-derived branches to DNA methylation, leading to failed amplification of one allelic gene in heterozygous DNA templates.

For field performance evaluation of root information, some different were found between two types of the root total length, root average diameter and root volume. Zhang et al. (2015) found that *Argyranthemum* plants are recovered from cryopreserved shoot tips showed a range of differences in vegetative growth and root information compared to in vitro culture plants. Wang et al. (2021c) found that *Allium* plants are

recovered from cryopreserved shoot tips showed no significant differences in rooting and vegetative growth compared to in vitro culture plants. In cryopreservation, especially when using shoot tip cryopreservation methods, only the apical meristem is often preserved. After recovery, the root meristems and root systems might need to be regenerated from the shoot apex, whereas in non-cryo materials, root systems might already be primed or pre-initiated. This could lead to a delay or reduced root growth in cryopreserved materials compared to non-cryo materials. In this study, the field performance of Raspberry ‘Stiora’ plants derived from cryopreserved shoot tips was compared with those from in vitro cultures (the control). The field performance of vegetative growth, including plant height, fully opened leaves, fresh weight, dry weight, and leaf area, was found to be same between two types. Although there have been significant differences in the field performance evaluation of various germplasm resources after cryopreservation, no negative evaluation has been reported following cryopreservation. The differences between cryo-derived and in vitro culture may be attributed to plant species/genotypes (Wang and Perl 2006; Kulus and Zalewska 2014; Wang et al. 2021b; Bettoni et al. 2024).

In conclusion, droplet vitrification for the cryopreservation of raspberry in vitro shoot tips was successfully developed in this present study and can be used for in vitro long-term storage of raspberry germplasm. Cryopreservation of Raspberry ‘Stiora’ shoot tips do not comprise field performance and cause genetic variation.

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Author contributions Xiao-Yan Ma: methodology, data collection, analysis, manuscript writing - original draft; Lu Tong: data analysis; Øyvør Stensbøl: methodology; Dag-Ragnar Blystad: funding acquisition and review; Qiao-Chun Wang and Dong Zhang: review and editing; Zhibo Hamborg: funding acquisition, methodology, writing, review, and editing. All authors have read and agreed to the published version of the manuscript.

Data Availability All data generated or analyzed during this study is included in this published article and its supplementary information file.

Declarations

Competing interest The authors declare that there was no competing financial interest or personal relationship that could influence the present study.

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