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Methodology for co-cryostorage of virus isolates in their host plants and relevant data

METHODOLOGY

2023

The methodology is the output of the project “Healthy berries in a changing climate: development of new biotechnological procedures for virus diagnostics, vector studies, elimination and safe preservation of strawberry and raspberry which benefits from a €1,477,000 grant from Iceland, Liechtenstein and Norway through the EEA Grants and the Technology Agency of the Czech Republic (TO01000295). The methodology was reviewed on December 19, 2023, a contract No. 18/2023 regarding the application of the methodology was concluded in accordance with the provisions of Section 1746, subsection 2 of Act No. 89/2012 Coll., Civil Code. As a certification body, the Ministry of Agriculture of the Czech Republic, issued Certificate No. MZE-73887/2023-13113 on the recognition of the methodology on December 22, 2023.

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Alois Bilavcik *et al.*

Methodology for co-cryostorage of virus isolates in their host plants and relevant data

METHODOLOGY

Issued by Crop Research Institute, Prague, Czech Republic
2023

Co-cryostorage of virus isolates in their host plants and relevant data

Currently, the nursery production of plant material emphasizes the production of healthy, virus-free material. For this reason, it is necessary to have reference plant materials in which the virus is present. Some types of viruses are difficult to maintain outside the plant host. Because of the potential transmission to outdoor plantings, it is necessary to keep infected plants in special cultivation areas, and net-houses, which brings considerable financial and labour costs. Therefore, a methodology was developed in which the virus status in plant material is analysed, the infected material can be stored using cryopreservation in liquid nitrogen and then regenerated *in vitro*, and relevant data in GeneEver cryobox can be stored.

Keywords: conservation; genetic resources; virus; *Rubus idaeus* L.

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Content:

I. Aim

The aim of the methodology is to develop a procedure for long-term preservation of infected raspberries by storing dormant buds at ultra-low temperatures in a viable state, detection of viruses using modern biotechnological methods and a system for preserving the relevant data in GeneEver cryobox.

II. Introduction

Raspberry (*Rubus idaeus* L.) is a valuable berry plant that is grown both in the Czech Republic and in other temperate countries. As raspberry is a perennial crop, cultivars and elite breeding lines must be maintained, usually as a plant collection in the field or as *in vitro* cultures. Preservation in situ and *ex situ* conditions (field collection) has significant spatial requirements, especially in the case of the repetition of important genotypes in multiple locations to reduce the risk of their loss and preserve varieties. In addition, many pathogens can attack raspberry plant collections grown in the field (Martin *et al.*, 2013). Since, from a breeding point of view, the varieties of fruit crops are clones whose seeds are heterozygous; there is no possibility of their simultaneous preservation in seed form. In addition, only a limited number of viruses can be transmitted through the seeds. *In vitro* cultures can be used as a backup for keeping selected clones, but there is an increased potential for mutations, for example, as a result of undetected indirect organogenesis or a high concentration of growth hormones in the culture medium and the loss of the monitored virus isolate. *In vitro* culture also requires regular subculturing, during which losses may occur more often due to contamination or technical problems (Lambardi *et al.* 2006; Kovalchuk *et al.* 2009). Therefore, this method cannot be considered a long-term safe way of storing genetic material. Cryopreservation is currently considered a prospective method for the long-term preservation of particular genotypes and viruses in their host plants. Similarly, as it is important to preserve the selected genotype of the fruit tree, so is their characterization before and after cryopreservation necessary for specific virus isolates - the particular virus isolates to be in the host plants subjected to the cryostorage. Cryopreservation laboratories work purposefully to preserve the gene material, but equally important is the preservation of the related data that needs to be stored together with it. GeneEver solves this challenge by combining state-of-the-art cryopreservation technology with future-proof long-term data storage as one holistic solution.

III. Description of the methodology

1. Characterisation of virus in plant material

Raspberry bushy dwarf virus (RBDV) is a member of the genus *Idaeovirus* (family *Mayoviridae*) and recently was renamed as *Idaeovirus rubi* (https://gd.eppo.int/taxon/RBDV00). The virus has non-enveloped isometric particles, about 33 nm in diameter (Barnett and Murant 1970). Virion preparations consist of three distinct species of linear, positive-sense, single stranded RNA, measuring around 5.5 kb (RNA-1), 2.2 kb (RNA-2), and 1 kb (RNA-3) (https://ictv.global/report_9th/RNApos/Idaeovirus).

In nature, the host range is not limited to the *Rubus* species. RBDV has been found to cause an infection in grapevines (Mavrič *et al.*, 2003; Jevremović and Paunović, 2011) and sweet cherry (Çağlayan *et al.*, 2023). Furthermore, RBDV was experimentally transmitted to more than 50 herbaceous host species via mechanical inoculation (Jevremović and Paunović 2011). The virus occurs in all tissues of the plant, including seed and pollen. Pollen and seed transmission are the only known methods of natural spread. The virus is prevalent worldwide in regions where raspberry is cultivated. In the Czech Republic, RBDV was detected in a range of raspberry cultivars sampled from commercial plantings and germplasm collections (Špak *et al.*, 1998).

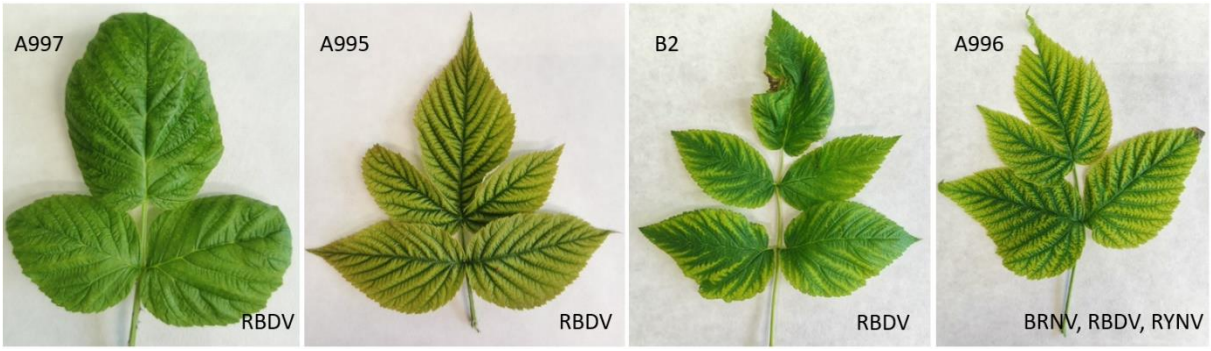

Figure 1 Symptoms of interveinal yellowing ranging from mild chlorosis (A997) to severe yellowing (A995, A996, B2) on leaves from various shrubs of *Rubus idaeus* cv. Enrosadira infected with raspberry bushy dwarf virus (RBDV) alone or in co-infection with black raspberry necrosis virus (BRNV) and Rubus yellow net virus (RYNV). (Franova)

While infections in raspberry and blackberry are often symptomless, certain infected cultivars may be associated with “yellows disease”, *Figure 1*, and/or “crumbly fruit”, presenting the major economic challenge in raspberry production. Notably, black raspberry necrosis virus is the primary factor behind bushy dwarf disease in Lloyd George raspberry. Despite this, the coexistence of RBDV in plants significantly amplifies the intensity of the disease symptoms usually observed in the field, making it an integral component of the overall disease syndrome (https://ictv.global/report_9th/RNApos/Idaeovirus). Moreover, RBDV is frequently detected in coinfection with either raspberry leaf mottle virus or raspberry latent virus, leading to a reduction in cane growth, fruit firmness, and weight (Quito-Avila *et al.*, 2014).

Pollen transmission significantly contributes to the spread of the virus, and addressing the economic impact on fruit quality necessitates the use of virus-free stock materials. Numerous studies have explored techniques for eliminating RBDV from plant tissues. The virus's ability to infect meristematic cells in the apical dome is a key factor limiting the effectiveness of traditional approaches such as chemo-, cryo-, and thermotherapy (Mathew *et al.*, 2021;

Weber, 2016). Combinations of these methods have been reported to be more effective, with RBDV elimination efficiency in raspberries reaching up to 79% (Mathew *et al.*, 2021). Recently, the eradication of RDBV from infected grapevines was achieved using *in vivo* thermotherapy combined with *in vitro* meristem tip micrografting, resulting in the absolute elimination of RBDV among other coinfecting viruses (Miljanić *et al.*, 2022). Thus, keeping the virus within plant tissues could be just as problematic as the efforts invested in its elimination.

1. Sample collection for virus analysis

Young raspberry leaves collected during spring to autumn (May - September), sprouted leaves and raspberry plants grown under *in vitro* conditions are suitable for RNA isolation. Because RBDV can occur unevenly in plants, it is recommended to sample the entire shrub circumference (8-12 leaves), preferably from all cardinal directions, to obtain an average mixed sample. If possible, leaves with symptoms of viral disease should preferably be sampled. Samples taken and properly labelled should be kept in a cool place and transported to the laboratory as soon as possible.

In the case of dormant raspberry canes of the 'Polka' variety (internal designation 'Polka' Pol) plant material, one year old canes of 60 to 120 cm long, were taken from the production field in Berry servis, Ltd. Brezany, Czech Republic, at the end of January 2021. The canes were cut into 25 cm long segments and placed into water under laboratory conditions for sprouting for virus analysis.

2. RNA isolation

Young leaves, *Figure 2*, were used for the extraction of double stranded RNA (26. 2. 2021) according to Morris and Dodds (1979). The viral composition of the sample was analyzed by high throughput sequencing. The double stranded RNA (A503) was used for the preparation of the sequencing libraries with Zymo-Seq RiboFree Total RNA Library Kit (Zymo Research, Irvine CA, USA). After data processing (252 million reads), raspberry bushy dwarf virus (RBDV) was detected in the sample. The sequence of RBDV was deposited in the GenBank database under the accession number (Acc. No. OR888447).

Figure 2 Raspberry canes with sprouted leaves used for dsRNA isolation. (Franova)

For routine virus detection, a commercially supplied Ribospin ™ Plant kit (GeneAll Biotechnology, Co., Ltd.; Cat. No.: 307-150; supplied by Bohemia Genetics Ltd.) was used for RNA isolation. The RNA isolation was carried out in the dedicated facilities of the Laboratory of Plant Virology. The manufacturer's instructions were followed with slight modifications.

Workflow:

1. Weigh up to 100 mg of plant tissue from raspberry, then grind the sample to a fine powder using a mortar and pestle with liquid nitrogen and transfer the powder into a 1.5 ml microcentrifuge tube.
2. Add 400 µl of Buffer RPL to the 1.5 ml centrifuge tube and vortex vigorously.
3. Incubate 3 min at room temperature.
4. Transfer the lysate to an EzPure ™ Filter by pouring or pipetting.
5. Centrifuge at ≥ 10,000 g for 30 sec at room temperature.
6. Transfer the supernatant to a new 2 ml microcentrifuge tube.
7. Add 1 volume (usually 350 µl) of 70% ethanol to the tube containing supernatant and mix well by pipetting or inverting.
8. Apply the mixture to a Column Type W (blue ring).
9. Centrifuge at ≥ 10,000 g for 30 sec at room temperature. Discard the pass-through and reinsert the mini column back into the collection tube.
10. Add 500 µl of Buffer RBW to the mini column.
11. Centrifuge at ≥ 10,000 g for 30 sec at room temperature. Discard the pass-through and reinsert the mini column back into the collection tube.
12. Apply 70 µl of DNase I reaction mixture to the center of the mini column. Incubate at room temperature for 10 minutes. (To make DNase I reaction mixture, mix 2 µl of DNase I solution with 70 µl Buffer DRB per isolation. DNase I is sensitive to physical damage. Therefore, do not mix vigorously.)
13. Add 500 µl of Buffer RBW to the mini column and incubate for 2 min.
14. Centrifuge at ≥ 10,000 g for 30 sec at room temperature. Discard the pass-through and reinsert the mini column back into the collection tube.
15. Add 500 µl of Buffer RNW to the mini column.
16. Centrifuge at ≥ 10,000 g for 30 sec at room temperature. Discard the pass-through and reinsert the mini column back into the collection tube.
17. Repeat steps 15-16.
18. Centrifuge at ≥ 10,000 g for an additional 1 min at room temperature to remove residual wash buffer. Transfer the mini column to a new RNase-free 1.5 ml microcentrifuge tube.
19. Add 40 µl of nuclease-free water to the center of the membrane in the mini column and incubate at room temperature for 10 min.
20. Centrifuge at ≥ 10,000 g for 1 min at room temperature.
21. The purity and concentration of the isolated RNA are determined using a nanodrop. High quality RNA should have an A_{260}/A_{280} ratio within the range of 1.8-2.2.
22. Purified RNA is stored on ice for immediate analysis and transferred to -80 °C for long-term storage.

3. cDNA preparation

- Reverse transcriptase is used to prepare cDNA: M-MLV Reverse transcriptase, 200 U/µl (Invitrogen, Cat. No. 28025-013; supplied by Life Technologies Czech Republic Ltd.).
- A mixture of random primers-hexamers is used for cDNA preparation. Random Hexamer Primer (Thermo Scientific, Cat. No.SO142; supplied by Fisher Scientific Ltd.).
- A mixture of nucleotides is used for cDNA preparation: dNTP mix, 10 mM each (Thermo Scientific, Cat. No. R0192; supplied by Fisher Scientific Ltd.).
- The RNA extraction prepared using the Ribospin^{TM} kit is then used for cDNA preparation Ribospin ^{TM} Plant kit (GeneAll Biotechnology, Co., Ltd.; Cat. No. 307-150; supplied by Bohemia Genetics Ltd.).
- A maximum of 500 ng of isolated RNA is used for the preparation of cDNA.The prepared cDNA can be stored for a short time for further analyses (*e.g.* PCR) at 4 °C or at -20 °C for long-term storage.
- Preparation of cDNA was carried out in a dedicated area within the Plant Virology laboratory using a PCR box.

Workflow:

1. Prepare the required number of 0.2 ml microtubes in a tube holder according to the number of samples.
2. For each 10 µl reaction mix:
   a. 0.5 µl of random hexamer primers at a concentration of 200 ng/µl
   b. 0.5 µl of 10 mM dNTPs
   c. 1-3 µl of total RNA (the total amount of RNA in the reaction should not exceed 500 ng)
   d. RNAse free water up to a total volume of 6.5 µl
   Mix the mixture by pipetting.
3. The mixture is heated in a thermocycler at 65 °C for 5 minutes. After the incubation period, the tubes are cooled rapidly in a frozen cooler or on ice. The tubes are then briefly centrifuged for a short time.
4. Add:
   a. 2 µl of 5 × First-Strand Buffer
   b. 1 µl of 0.1 M DTT
   c. 0.5 µl (200 U) M-MLV reverse transcriptase
   Mix well by pipetting.
6. Place the samples in a thermocycler under the following program:
   a. Incubation at 37 °C for 2 minutes.
   b. Incubation at 25 °C for 10 minutes.
   c. Incubation at 37 °C for 50 minutes.
   d. Inactivation of reverse transcriptase at 70 °C for 10 minutes.
   e. Final cooling to 4 °C.
8. cDNA prepared in this way can be used directly as a PCR template (usually 1 µl) or it can be diluted 10-fold. cDNA is stored in a freezer at -20 °C.

4. Detection of RBDV by means of reverse transcription polymerase chain reaction (RT-PCR)

Primers RBDV_CRF/RBDV_CRR (5´-TTTTCTACGGCTGCTGGTCT-3´/ 5´- GCATGTCCCTCAGTTTCGAT-3´) were used for amplification of 370 bp long portion of the

RBDV replicase gene [James Hutton Ltd. (https://www.hutton.ac.uk)]. One µl of each cDNA preparation was added to 10 µl of PPP Master Mix (150 mM Tris-HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20, 5 mM $MgCl_2$, 400 µM of each dNTPs, 100 U/ml Taq-Purple DNA Polymerase; Top-Bio Ltd., Prague, Czech Republic), 7 µl PCR H_2O and 1 µL (final 0.5 µM of concentration) of each primer. The following conditions were used for amplification: denaturation step of 1 min at 94 °C followed by 35 cycles consisting of 15 sec at 94 °C, 15 sec at 55 °C and 1 min at 72 °C, then a final extension step of 7 min at 72 °C. The cDNA from RBDV positive samples (the A709, A805, A806, A951, B41 isolates) and no-template control (amplicon from PCR reaction without addition of template) were employed as positive and negative control in all PCR assays, respectively. Four microlitres of each PCR product was analysed by electrophoresis in a 1% agarose gel (prepared by mixing agarose with 1 X Tris-borate-EDTA buffer) supplemented with 1:10000 v/v GelRed stain (Biotium, Inc., Hayward, CA, USA) and DNA bands were visualized using a UV transilluminator.

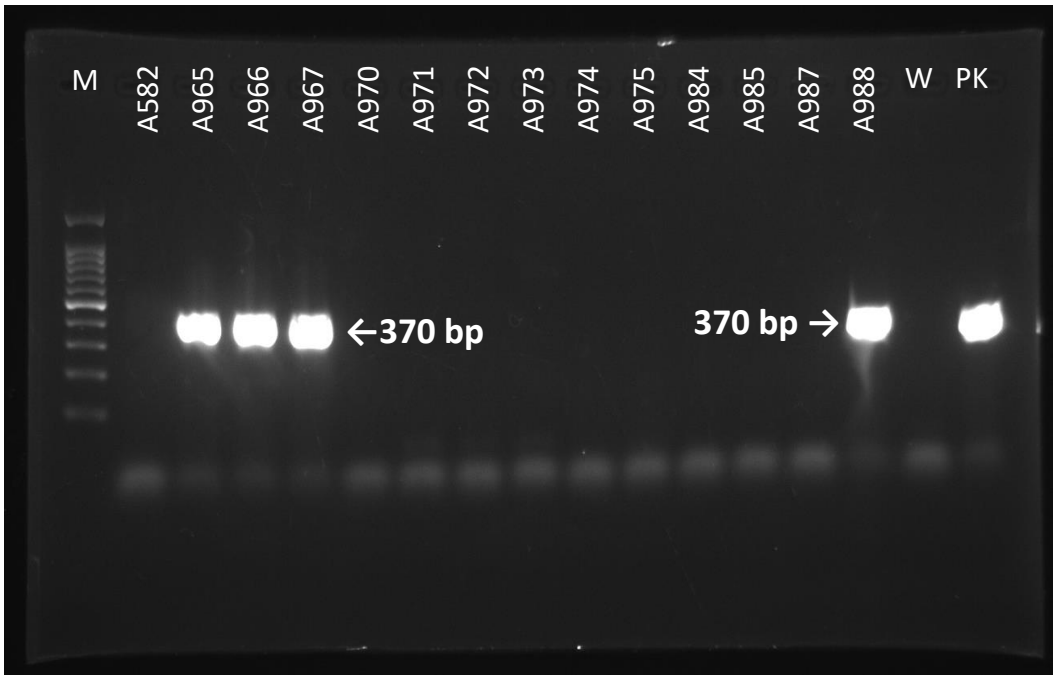

Figure 3 Illustration of the RBDV-specific RT-PCR assay in raspberry samples. W – no-template control, PC – positive control (RBDV isolate A951), M–100bp DNA ladder, from top to bottom: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp. The arrows indicate positive samples. (Franova)

5. Verification of the virus presence by Sanger sequencing

The PCR products (16 µL) of appropriate size were excised from 1.5% agarose gel or purified directly using ExpinTM Combo GP mini kit (GeneAll Biotechnology, Co., Ltd.; Cat. No.: 112-102; supplied by Bohemia Genetics Ltd.). The purified PCR products were Sanger sequenced from both directions (Eurofins Genomics, Luxembourg). The obtained sequences were quality- and primer-trimmed and then assembled by employing the Contig Express (a component of Vector NTI Suite 8.0 software) and compared with data available in GenBank using the World Wide Web service BLASTN (https://blast.ncbi.nlm.nih.gov).

2. Cryopreservation method

Sakai and Nishiyama (1978) were the first to successfully cryopreserve dormant raspberry buds by slow freezing from -5 °C to -45 °C before immersion in liquid nitrogen (LN). Reed and Lagerstedt (1987) used a cryoprotectant solution composed of polyethylene glycol, urea, and dimethyl sulfoxide (DMSO) for cryopreserving *in vitro* raspberry shoot tips. Reed (1988) used cold hardening of raspberry *in vitro* cultures to increase their survival. Currently, cryopreservation procedures are employed using vitrification methods, where vitrification (glassy state) is achieved in cryopreserved tissues with the help of glass-forming substances (Volk and Walters 2006; Zamecnik *et al.*, 2018). For many plants, the vitrification procedure based on PVS2 solution is mainly used to preserve their genetic resources (Sakai *et al.*, 1990). Due to the relatively high toxicity of this solution (Kim *et al.*, 2009) and especially with regard to the potential mutagenic effects of one of the components of the PVS2 solution, namely DMSO (Valencia-Quintana *et al.*, 2012), there have recently been efforts to use vitrification solutions without DMSO. One common cryoprotective solution without DMSO is PVS3, composed of 50% sucrose (w/v) and 50% glycerol (w/v) in water (Nishizawa *et al.*, 1993; Wang *et al.*, 2020). Many *Rubus* species are vegetatively propagated and are subject to infection by more than 30 known viruses during development, propagation, and fruit production (Martin *et al.*, 2013; Koloniuk *et al.,* 2023). The problem of using *in vitro* vitrification methods for the conservation of viral isolates in their host plants is the possibility of eradicating the conserved virus from the plant host. The *in vitro* culturing and especially cryopreservation could act as a therapy for the viral isolate (Grout 1999; Cheong *et al.*, 2014; Wang *et al.*, 2018; Zhang *et al.*, 2019; Bhat and Rao, 2020). The principle of the therapy methods is to regenerate the healthy meristematic part of the plant. For *in vitro* culture, the meristematic part is dissected from the plant and transferred to regeneration medium. Cryotherapy can act as a “cryoknife” - the regeneration of meristems after LN exposure (using cryopreservation methods). The older differentiated tissues are “cut out” of the meristematic because they do not survive LN treatment and only healthy tissues regenerate. A combination of *in vitro* culture technique with cryopreservation can eliminate the screened virus and the host plants can be virus free (Kushnarenko *et al.*, 2017).

The above-mentioned reasons led us to test the dormant bud cryopreservation technique for the conservation of raspberry genotypes with virus isolates and evaluate the possibility of regenerating the regrowing plants *in vitro*. The dormant bud cryopreservation technique was based on the efficient protocols developed for apples in the USA (Stushnoff and Seufferheld, 1995; Towill *et al.*, 2004; Towill and Bonnart, 2005). With the use of the developed protocol, a great number of genotypes have been cryopreserved (Forsline *et al.*, 1998; Towill *et al.*, 2004). Further investigation into mechanisms and physiological aspects of cryopreservation of dormant buds has continued in Europe *e.g.* Denmark (Toldam-Andersen *et al.*, 2007, Vogiatzi *et al.*, 2011), Germany (Höfer, 2015), and in the Czech Republic (Bilavcik *et al.* 2015, 2021). The cryopreserved dormant buds were successfully regenerated *in vitro* in *Morus* (Oka *et al.*, 1991), *Diospyros* (Matsumoto *et al.*, 2004) or blackcurrant (Rantala *et al.*, 2019).

1. Chemicals and cultivation means

The following list contains all the chemicals needed for cryopreservation of dormant raspberry buds.

Dehydrating and rehydrating means:

- silicagel
- white peat

- destilled water

Cooling and storage medium:
- liquid nitrogen

Cultivation medium:
- MS typ medium (Murashige and Skoog, 1962; Dziedzic and Jagla, 2013)
- phytohormones
- agar
- sucrose
- destilled water

Cooling and storage medium:
- liquid nitrogen

2. Small accessories

The following tools are needed to implement the method of cryopreservation of dormant buds of raspberries:

Working with dormant buds in outdoor conditions and storing them before freezing:
- garden shears and knife
- string, name tags, PE bags

Frost dehydration:
- garden shears, scissors, tweezers
- nets for dehydrating buds, PE bags
- baskets for storing nets in the freezer box
- balances

Cryoconservation:
- centrifuge tubes 50 ml (CLP, Biologix)
- aluminum sheets (20 x 6 x 0.05 mm)
- styrofoam containers, paper boxes for a Dewar container
- PE bags, name tags

Regeneration:
- flow-box
- autoclave
- pH-meter
- microwave
- cultivation flasks, Petri dishes, tweezers, scissors, scalpels

Figure 4 Preparation of segments from dormant raspberry canes for frost dehydration. (Bilavcik)

3. Plant material

The source material for cryopreservation of dormant buds of raspberry was one-year-old dormant canes. The optimal length of canes is from 60-120 cm (even longer). The canes should be at least 6 mm in diameter, well ripened and without signs of damage by biotic or abiotic agents, *Figure 4*. The raspberry varieties 'Sanibelle' and 'Polka' were used in the experiments. Because of the accessibility of the infected plant material, the variety 'Sanibelle' was used for testing the cryopreservation procedure and the variety 'Polka' was used for testing the introduction of the RBDV host plant from dormant buds to *in vitro*. The canes of the 'Polka' variety were taken from Berry servis, LTD., Brezany, Czech Republic, at the end of January 2021, and the canes 'Sanibelle' from the hotbed in Crop Research Institute, Prague, Czech Republic, at the end of January 2023. The experiments were set up to have a consistent procedure of the co-cryostorage of virus isolates in their host plants. The harvested plant material was stored in plastic bags at -3.5 °C until conducting the experiments (14 to 30 days).

4. Cryopreservation Workflow

The procedure of cryopreservation of dormant raspberry buds can be divided into four successive steps:

a) Frost dehydration of dormant buds
b) Cryopreservation of frost dehydrated dormant buds
c) Thawing and rehydration of dormant buds
d) *In vitro* escape

a) Frost dehydration of dormant buds

The dormant 'Sanibelle' canes were cut into nodal segments with one bud in the middle and placed at -4 ° C in a freezer, *Figure 5*. At this temperature, they were frost dehydrated for 4-7 weeks until they reached 25 to 30% of water content. The water content was determined gravimetrically on a fresh weight basis after drying a random sample of five segments at 85 °C for a constant weight.

Figure 5 Frost dehydration of raspberry nodal segments in a freezer at -4 °C. (Bilavcik)

b) Cryopreservation of frost dehydrated dormant buds

After frost dehydration, a sufficient number of nodal segments, from 20 to 25 segments were frozen in 50 ml tubes covered with aluminium foil, Kartell Conical Grad Test Tube, Kartell S.p.A., Italy, with a two-step cryoprotocol. In the first step of the cryoprotocol, the temperature was lowered from -4 ° C to -30 ° C (cooling rate 1 ° C h^{-1}) in a computer-controlled freezer and after equilibration for 24 hours; the tubes were immersed in liquid nitrogen, the second step of the cryoprotocol. The batch of 120 buds was frozen and stored in the cryobank storage, Dewar flask LS4800 Taylor Wharton, USA.

c) Thawing and rehydration of dormant buds

From the cryopreserved batch, a set of 20 nodal segments for evaluation of the freezing protocol and further regeneration studies were used. The nodal segments were placed at +4 °C for survival and regeneration evaluation and allowed to slowly thaw overnight spontaneously. After thawing, the buds were placed into a moist white peat for rehydration for 14 days at +4 °C. After rehydration of the 'Sanibelle' cryopreserved buds and the 'Polka' dormant buds from the field, both samples were subjected to a sterilisation procedure for *in vitro* introduction.

d) *In vitro* escape

First, the buds were subjected to 20 min washing under the tap water and then the sterilisation was done according to Välimäki *et al.* (2022). Buds were treated for 30 min with 20 g l^{-1} sodium dichloroisocyanurate (NaDCC) in agitation, next they were kept in 70% EtOH for 15 min. The buds were finally rinsed with sterilized water three times and left in water until preparation (up to 1 h). The buds with a part of the twig, approximately 1 cm long, *Figure 6*, were placed on a semisolid MS medium modified according to Dziedzic and Jagla (2013), 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 7 g l^{-1} agar and put in standard cultivation conditions in a cultivation room (24 °C, 16/8 photoperiod), *Figure 7*. The control plants were introduced *in vitro* conditions by the same sterilisation procedure. The sprouting buds were evaluated for bacterial and fungal contaminations and contamination free 'Polka' plants, *Figure 8*, were multiplied and evaluated for internal virus retention.

Figure 6 Disection of the dormant bud from the cryopreserved nodal segment. (Bilavcik)

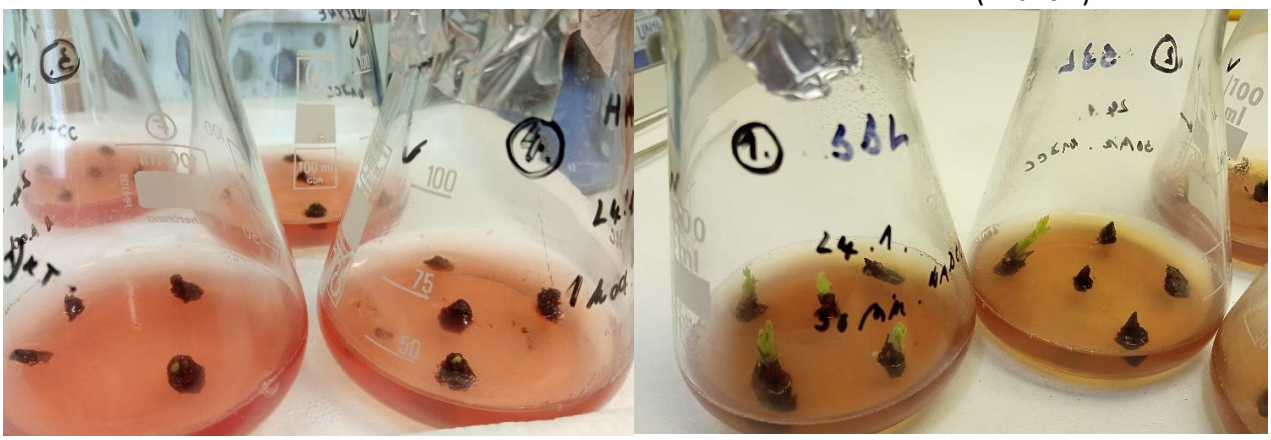

Figure 7 Regenerating cryopreserved dormant raspberry buds of variety 'Sanibelle' *in vitro*. Left – after 10 days, right after 13 days after rewarming from cryopreservation. (Bilavcik)

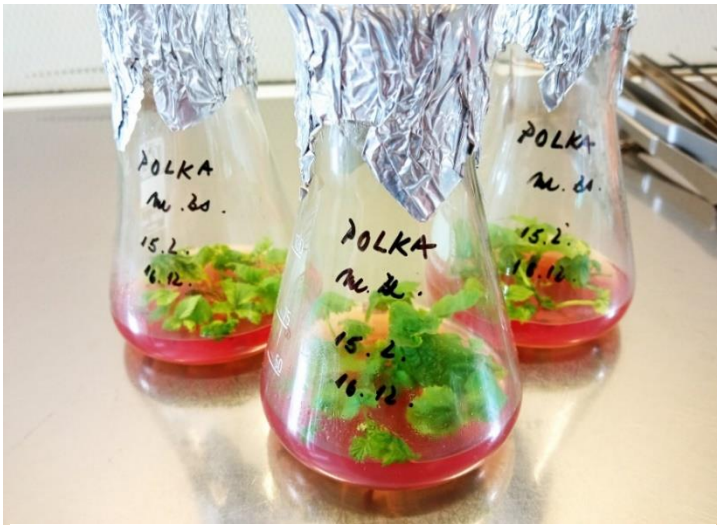

Figure 8 Regenerating dormant raspberry buds of variety 'Polka' *in vitro*. (Bilavcik)

3. Relevant data storage in GeneEver cryobox

To be able to utilize the biological material in the future, it is also necessary to have access to the information related to the plant. Information that describes the seeds, how they are grown, the health status of the plant, how to utilise the crops, photos etc. Thus far, the information and the plant material have been stored in different locations. The data could be stored on paper, hard drives, CDs or similar, where there is no direct connection with the biological material. If the data is digital, it has to be transferred from one digital storage medium to the next due to the short lifespan of hardware in order to survive with the plant. GeneEver combines unique biotechnology with unique data storage technology and offers long term co-preservation of seeds and living plant material combined with its associated data and information. This is a new, comprehensive solution that guarantees access to fresh and healthy food coupled with information on how it can be cultivated in the future. Piql has developed a new storage technology that can secure access to the information concerning the biological material in the future. Piql solves the challenges related to developments in technology with a unique, migration free and "selfcontained" storage solution for digital and visual (i.e. human readable) information. The storage medium has unique long-term attributes that become even better when the storage medium is stored in a cold and dry climate (*e.g.* at Svalbard), *Figure 9*.

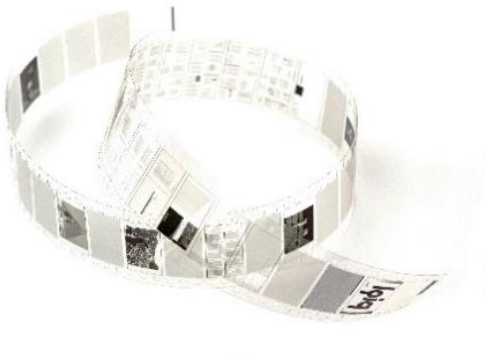

Figure 9 PiqlFilm with all information and associated data stored. (Bjerkestrand)

Figure 10 Exapmle of cryo-data on the Piql film. (Bjerkestrand)

Migration free implies that the data does not have to be transferred from one storage medium to another due to obsolescence of the storage medium itself or related hardware or software. With Piql's solution, the data is written to Piql's storage medium once – and it will stay there until someone needs to read it back, *e.g.* when other storage systems are no longer accessible or fail. Today's storage technology typically has a lifespan of 5 years. Who has a computer older than 5 years?

"Self-contained" implies that all the information needed to retrieve the data in the future is contained on the storage medium in clear text, making it possible to read and understand how to use future "off-the-shelf" technology to read back the data, Figure 10. On conventional storage technology (*e.g.* hard disk, CD or magnetic tape), there is no such clear text information available, and you would need specific technology to read the data back.

Reading back data from Piql's technology in the future can be done independently of specific vendors. One would only need a light source, a digital camera and a computer of some sort. If these three things (at least one of them) exist in the future, the data will always be retrievable .

The CryoBox, see *Figure 11*, is designed in a way that allows the biological material and the related information to be cryopreserved together. This way, one does not need to keep a separate archive to be able to access the information in the future. GeneEver has developed a separate service, GeneEver Connect, where the customer can choose and manage the information and data they want stored on piqlFilm. GeneEver Connect is a good tool for finding what has been deposited on the cryotank, and has been developed so that it contains database solutions for those who want it. Read more about GeneEver at www.geneever.com.

Figure 11 CryoBox with co-storage of cryopreserved biological material and information stored on piqlFilm, all together in the same box. (Bjerkestrand)

IV. Comparison of novelty procedures

The proposed methodology brings a new procedure that enables the safe preservation of viruses in living raspberry plant material, in their dormant buds. Until now, a similar comprehensive methodical procedure for evaluating the virus status in the raspberry plant material and preserving viruses in living plants of raspberry based on the cryopreservation of dormant buds with the relevant data stored has not been published in the Czech Republic yet. The safety conservation of the virus pool of the raspberry can be carried out using dormant bud cryopreservation and *in vitro* escape. Cryopreservation using *in vitro* cultures is based in principle on a different method of cryopreservation – on ultra-fast freezing of very small parts of the plant, that can be virus free and such a method is not generally suitable for viruses in plant conservation.

V. Description of the application of the methodology

The Ministry of Agriculture of the Czech Republic will be the user of this methodology through the "National Program for the Conservation and Use of Genetic Resources of Plants, Animals and Microorganisms Important for Nutrition and Agriculture". This methodology will make it possible to evaluate virus status of raspberry plants and safely cryopreserve the selected plant material in dormant buds with known virus present for future regeneration of the infected plant material and relevant data storage.

VI. Economic aspects

The calculation of costs for the implementation of the procedure for co-cryostorage of virus isolates in their host plants and relevant data specified in the methodology depends on whether the entire operation for the *in vitro* laboratory and cryopreservation are newly introduced or whether this cryopreservation methodology is only implemented in the existing operation of the *in vitro* laboratory. In the event that the laboratory already uses *in vitro* culture technology, the cost of introducing a new methodology is practically only the purchase of a Dewar container for sample storage, liquid nitrogen and a programmable freezer.

Cryopreservation of virus isolates in their host plants can be used for the safe preservation of desired virus isolates that cannot be kept without the life plant host. This method can be used either as a backup for collections or as a main procedure of the collection. In the long term, however, the cryopreservation method is more advantageous both financially and in terms of virus isolate stability. In the case of cryopreservation using *in vitro* escape, the costs of working with tissue cultures in aseptic conditions are significant equipment for sterilization (autoclaves, hot air sterilizers), culture boxes, laminar boxes, laboratory equipment for the preparation of cryoprotective solutions. These costs are in the order of 750,000 CZK in the case of a newly furnished one. However, this equipment is often already present in laboratories dealing with *in vitro* cultures. Furthermore, storage space in Dewar vessels is needed. The price of one storage Dewar container for storing hundreds of virus isolates, depending on the success of regeneration, is approximately 150,000 CZK. In the case of keeping 200 isolates in one Dewar container, the material price for the introduction of one genotype is approximately 3,000 CZK including cryotubes, a box and liquid nitrogen for freezing. Personnel costs are approximately 15,000 CZK per item. When freezing the maximum technically manageable 30 isolates at a capacity per year (1.0 full-time hours), the total annual costs can be estimated at 453,000 CZK. If the Dewar storage container is fully loaded with 200 isolates, with 2 to 3 cryotubes per isolate, the annual liquid nitrogen consumption is approximately 20,000 CZK, *i.e.* 670 CZK per genotype. The method of cryopreservation is economically advantageous, especially in the case of a long-term preservation strategy, when, for example, compared to the method of cultivation of the host plants in net houses, it saves storage capacity and preserves material, which can be further directly used in biotechnological applications or transferred to *ex vitro* conditions. The preservation of a spectrum of virus isolates with co-storage of the relevant data is significant by reducing the risk of losing individuals or the data relating to the isolates, which is the main benefit of this methodology for users.

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VIII. List of publications that preceded the methodology

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IX. Dedication

The methodology is the output of the project "Healthy berries in a changing climate: development of new biotechnological procedures for virus diagnostics, vector studies, elimination and safe preservation of strawberry and raspberry" which benefits from a €1,477,000 grant from Iceland, Liechtenstein and Norway through the EEA Grants and the Technology Agency of the Czech Republic (TO01000295).

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