

CRYOTHERAPY IN RASPBERRY (*RUBUS IDAEUS* L.) AS A POTENTIAL METHOD FOR OBTAINING VIRUS-FREE PLANTS AND GENETIC COLLECTIONS

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ABSTRACT

Red raspberry (*Rubus idaeus* L.) is a small-fruit crop of key economic importance, widely cultivated for fresh consumption and processing, and valued in traditional medicine. Vegetatively propagated material is subject to increased pathogen pressure, especially viruses, viroids, and phytoplasmas, leading to growth suppression, yield losses of up to 50%, and heightened susceptibility to secondary infections. Ensuring healthy, pathogen-free planting stock is therefore a priority in production and breeding. This review synthesizes current knowledge on cryopreservation methods applicable to raspberry, with particular emphasis on their suitability for virus eradication (shoot-tip cryotherapy) and identifies the technical parameters that determine procedural success. Cryopreservation and cryotherapy protocols based on vitrification (including variants based on plant solution vitrification and droplet vitrification) and encapsulation-dehydration were analyzed. The focus was on shoot apical meristems as the starting material and on combining cryotherapy with other sanitation procedures, for example thermotherapy. Among the available approaches, the highest post-thaw regeneration rates were obtained with vitrification protocols and with encapsulation-dehydration. Efficiency increases when cryotherapy is combined with thermotherapy. Success is strongly influenced by explant size and physiological state, preculture conditions (for example sucrose concentration), cryoprotectant composition and exposure time, and cooling and warming profiles. Beyond its role in the long-term conservation of genetic resources, cryopreservation is an effective tool for sanitizing raspberry material. Shoot-tip cryotherapy enables the production of virus-free plants, which is crucial for establishing healthy plantations and breeding programs. Incorporating standardized protocols into nursery certification systems may accelerate the dissemination of clean stock and, in the long term, reduce production losses. The evidence reviewed confirms that optimized cryopreservation protocols provide a reliable framework for both long-term conservation of germplasm and the regeneration of healthy raspberry planting material.

Key words: cryotherapy methods, cryotherapy efficiency, cryostorage, virus eradication, vitrification

INTRODUCTION

Raspberries, caneberries belonging to the genus *Rubus*, are of great economic importance as a food source and have been used in medicine for centuries (Kim et al. 2016). A large share of global raspberry production is concentrated in Europe, with Poland and Serbia playing a key role in cultivation and breeding (Wróblewska et al. 2019).

The viral challenge in raspberry cultivation

Raspberry plantations are highly vulnerable to infections with obligate pathogens such as viruses, viroids, and phytoplasmas, which are passed down from generation to generation by vegetative propagation. Plantations established with virus-infected material quickly degenerate. Other pathogens can thrive on weakened plants, leading to significant drop in yield, often by up to 50%, and shortening

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the productive life of the plantation (Converse et al. 1987). More than 40 viruses and virus-like agents are known to infect *Rubus* species, including *R. idaeus* and *R. occidentalis* (Martin 2002). Therefore, the priority in raspberry cultivation is to start plantations with pathogen-free material (Bhojwani & Dantu 2013).

For this reason, raspberries intended for planting are often propagated in vitro. Virus infections may reduce the efficiency of clonal propagation and in vitro multiplication (Kaczmarczyk et al. 2012). For instance, in vitro micropropagation of raspberry ‘Malling Landmark’ infected with Tobacco streak virus (TSV), Tomato ringspot virus (TomRSV), or Raspberry bushy dwarf virus (RBDV) showed less than half the multiplication rate obtained from virus-free cultures (Tsao et al. 2000).

PRINCIPLES OF CRYOTHERAPY VS. CRYOPRESERVATION

Cryobiotechnology serves two distinct functions: cryopreservation and cryotherapy. Cryopreservation is the process of preserving biological functions by freezing and storing living material below -80°C , typically at or near liquid nitrogen temperature (-196°C) (Feng et al. 2013). This method is primarily used for the long-term conservation of genetic resources in gene banks.

Cryotherapy, on the other hand, is the specific application of cryopreservation to eliminate pathogens from plant tissues. This innovative method is considered a modern biotechnological tool for producing pathogen-free plants (Wang et al. 2005). The mechanism of cryotherapy is based on the differential response of various cell types to freezing. Viruses are unevenly distributed throughout plants, and the highest concentrations typically occur in older, differentiated cells.

Cells in the upper apical dome (AD) and the youngest leaf primordia are small, poorly vacuolated, and have a high nucleocytoplasmic ratio. These tissues are more likely to survive cryotreatment, whereas more differentiated cells (often virus-infected) are preferentially killed (Wang et al. 2006, 2008). This selective survival underpins the

use of shoot-tip cryotherapy for pathogen eradication in vegetatively propagated crops.

These phenomena are exploited in cryotherapy, where the apical shoot tips are excised and subjected to a cryopreservation protocol. By regenerating plants from these surviving, pathogen-free cells, it is possible to obtain a pure plant population (Wang et al. 2009).

The evolution of cryotherapy in plant biotechnology

Initially, cryopreservation research focused on long-term storage of plant material to preserve biodiversity (Helliot et al. 2002). A breakthrough came when scientists discovered that the thermal shock caused by freezing in liquid nitrogen could selectively destroy virus-infected cells while sparing healthy ones. Pioneering work in this field took place in the late 1990s and early 2000s. Early studies showed that freezing shoot tips in liquid nitrogen could be an effective tool for obtaining virus-free plants (Brisson et al. 1997). Research on various plant species, including grapevine and fruit trees, have shown that cryotherapy can be even more effective than traditional methods using meristem culture (Wang et al. 2006).

Cryotherapy and molecular mechanisms

In addition to the physiological differences between infected and meristematic cells already described, several complementary hypotheses have been proposed to explain virus eradication during cryotherapy. First, viruses are often unevenly distributed throughout the plant and often do not colonize the shoot apical meristem. Thus, after cryogenic treatment, surviving meristem cells regenerate virus-free plants (Wang et al. 2009). Second, the shoot apical meristem possesses an innate immune system. The transcription factor WUSCHEL (WUS) has been shown to suppress Cucumber mosaic virus (CMV) replication in Arabidopsis, providing experimental evidence that meristems are not only passively virus-free but also actively protected (Wu et al. 2020). Third, physical stress factors generated during cryopreservation, including intracellular ice formation, osmotic imbalance, and oxidative stress, selectively kill larger, vacuolated, and often infected cells. In comparison, smaller meristematic cells are better adapted to survive these stresses (Wang et al. 2009).

Fourth, some authors suggest that high concentrations of cryoprotectants may exert a chemotherapeutic-like effect, further reducing the survival of virus-infected cells. This hypothesis remains controversial and has not been directly confirmed (Marković et al. 2015). Finally, in cases where viruses penetrate the meristem, such as RBDV or Apple stem grooving virus (ASGV), cryotherapy alone often proves insufficient. Combining thermotherapy with cryotherapy has yielded much higher success rates, with approximately 30–35% of regenerants free of RBDV, as confirmed by ELISA and RT-PCR (Wang et al. 2008).

The molecular mechanisms of cryotherapy involve the selective destruction of virus-infected cells while protecting and regenerating small, healthy meristematic cells. This phenomenon results from the physiological and molecular differences between these two cell types (Reed 2001).

A key element of cryotherapy is the differential susceptibility of cells to freezing damage. Most plant cells, including those infected with viruses, contain large vacuoles and are highly hydrated. During freezing, the water in these cells crystallizes, leading to mechanical rupture of the cell membranes and cell death (Kaviani 2011). Furthermore, this process can induce osmotic stress, which exacerbates damage. Meristematic cells at the shoot apex, on the other hand, are much more resistant to extremely low temperatures.

Cell resistance factors

Small size and dense cytoplasm

Young, immature meristem cells do not have large vacuoles, and their dense cytoplasm is rich in substances that prevent the formation of intracellular ice crystals.

Natural cryoprotectants

Meristematic cells are naturally capable of accumulating substances such as sugars, which lower the freezing point and promote vitrification – the transformation of water into a glass state, protecting them from damage (Nagel et al. 2024).

Response to oxidative stress

Cryopreservation induces oxidative stress through the accumulation of reactive oxygen species (ROS).

However, meristematic cells have more efficient defense mechanisms to neutralize these harmful substances. Increased production of antioxidant enzymes (e.g., superoxide dismutase) is crucial for the survival of these cells. Virus-infected cells, already weakened by viral replication, are more susceptible to oxidative stress-induced damage (Mathew et al. 2021).

Evidence of the effectiveness of cryotherapy

Recent reviews have concluded that cryotherapy has been successfully used for virus eradication in numerous plant species across a broad spectrum of horticultural crops (Wang et al. 2009), including grapevine, citrus, plum, banana, and various *Rubus* species. The effectiveness of cryotherapy depends on the virus species and its ability to colonize meristematic tissues. For example, RBDV in raspberry and ASGV in apple are highly invasive in meristems and therefore resistant to cryotherapy alone. However, combining heat treatment with cryotherapy significantly improves eradication efficiency in these cases (Wang & Valkonen 2009). These findings support the hypothesis that cryotherapy is not a universal solution but rather an effective, complementary approach that can be optimized through integration with other sanitation methods.

Studies have demonstrated the effectiveness of cryotherapy in eliminating viruses from various plant species. For example, cryotherapy of virus-infected plum rootstock ‘Fereley-Jaspi’ resulted in 45–60% virus-free plants (Brison et al. 1997). Similarly, in grapevines infected with Grapevine virus A (GVA), approximately 96% of plants regenerated from cryopreserved shoot tips were virus-free (Wang et al. 2003). It is believed that the destructive effect of cryopreservation on viruses is caused not only by the freezing process itself but also by the so-called chemotherapeutic effects of cryoprotectants (Marković et al. 2015).

A valuable study by Mathew et al. (2021) showed that although cryotherapy alone cannot completely eliminate RBDV from raspberries, the combination of thermotherapy and cryotherapy proved highly effective, producing approximately 35% virus-free regenerants. This suggests that some viruses may be more resistant and require a multifaceted approach for complete elimination.

COMPARISON OF TRADITIONAL METHODS WITH CRYOTHERAPY

Traditional methods are based on several principles. Thermotherapy involves exposing plants to elevated temperatures (e.g., 37–40 °C) to inhibit virus replication (Reed 2001). However, this process can be time-consuming and may damage plant tissues, often leading to low regeneration rates (Mathew et al. 2021). Similarly, chemotherapy uses antiviral compounds in the culture medium, but its effectiveness varies widely depending on the virus and the chemical used. This method also carries a risk of phytotoxicity (Wróblewska et al. 2019). The most common method, shoot tip culture, relies on the fact that meristematic tissues are often virus-free (Brison et al. 1997).

Cryotherapy, on the other hand, combines the best aspects of these techniques, offering effective and relatively rapid virus elimination. Similar to shoot tip culture, it targets virus-free meristematic cells in the shoot apex, but utilizes thermal shock for selection. This has a high success rate in virus eradication. Compared to thermotherapy, the process is much faster, reducing the risk of long-term stress in plants. However, the main challenge remains optimizing complex cryopreservation protocols, the success of which depends largely on the plant genotype and virus type (Kaviani 2011).

GENERAL CHARACTERIZATION AND CLASSIFICATION OF CRYOPRESERVATION METHODS

Slow freezing and simple freezing

Cryopreservation was developed in the 1970s and 1980s. The first methods developed were classical slow freezing and simple freezing (Bettoni et al. 2024). In the initial phase of both methods, plant material (shoot tips, embryos, and cells) was placed in a straw or cryotube and exposed to cryoprotectants (DMSO, glycerol, ethylene glycol, and sucrose). The differences between the methods lie in the type of freezing (Fig. 1, Table 1).

The slow freezing method involves controlled cooling of plant material in a programmable freezer. Plant material is placed in cryoprotective solutions, equilibrated (often to 0 °C), and then cooled within the range of 0.5–1.0 °C per min (e.g., 0.8 °C per min) to approximately –40 °C, and then rapidly immersed and storage in liquid nitrogen (Chang & Reed 1999; Nagel et al. 2024).

In the slow cooling method for raspberry cryopreservation explants are precultured before cooling (Reed & Lagerstedt 1987). They are transferred to modified MS medium for 48 h (Gupta & Reed 2006). The medium is supplemented with double-strength EDTA-Fe, 4.4 µM BA, 0.49 µM IBA, and 5% DMSO (Reed 2008). The explants are then immersed in cryoprotectants. The solution prepared based on the MS medium described above is supplemented with 10% PEG, 10% glucose, 10% PGD, and 5% DMSO (Sakai & Engelmann 2007). Cryoprotected explants are cooled to 0 °C for approximately 30 minutes, then slowly (at a rate of 0.5 °C per min) to –35 °C (Roque-Borda et al. 2021), to –40 °C (0.8 °C per min), or to –45 °C (0.3–0.5 °C per min), and then (10 °C per min) to –75 °C (Ukhatova et al. 2017). Once the appropriate temperature is reached, the explants are immersed in liquid nitrogen.

Vitrification

The vitrification method was developed by Sakai et al. (1990), and the procedure has been applied to many plant types (Matsumoto et al. 1994). Vitrification is based on a physical process in which a highly concentrated cryoprotective solution cooled to very low temperatures. It eventually solidifies, forming a metastable glass without undergoing a crystallization step (Pritchard 2007). An important requirement for vitrification is that the plant material (shoot tips and cells) in the cryoprotective solution be dehydrated to a degree that prevents lethal damage caused by cooling and subsequent warming before transfer to liquid nitrogen.

Each vitrification protocol can be divided into the following stages: preconditioning, osmoprotection with loading solution, incubation in PVS2 or PVS3, and post-liquid nitrogen treatment (Popova et al. 2015). For raspberry, during preconditioning, shoot tips are placed in a medium containing 5% DMSO for 48 hours. Dehydration is then performed by placing the plant material in a loading solution (LS: 2 M glycerol, 0.4 M sucrose) for 20 minutes (Roque-Borda et al. 2021). In the following stage, the plant material should be preserved in a cryoprotectant solution. In the presented method, the cryoprotectant solution PVS2 [30% (v/v) glycerol, 15% ethylene glycol, and 15% DMSO in liquid MS medium with 0.4 M sucrose] was applied for 20 minutes at 25 °C (Roque-Borda et al. 2021).

Encapsulation-dehydration

The encapsulation-dehydration method is based on the elimination of cell water via osmotic and evaporative dehydration to obtain the same state. In this method, plant material (shoot tips, somatic embryos, or callus cells) is encapsulated in alginate beads. These capsules are prepared with 3% (w/v) low viscosity alginic acid dissolved in liquid MS medium without added calcium and adjusted to pH 5.7. Capsule polymerization is performed in liquid MS medium supplemented with 100 mM CaCl₂ and 0.4 M sucrose for 20 minutes. In the next stage, to increase intracellular solute concentrations and facilitate desiccation, the plant material is incubated in MS medium containing a higher sucrose concentration. Desiccation is continued by placing alginate capsules on silica gel or subjecting the encapsulated explants to air (Feng et al. 2013). Before immersion in liquid nitrogen, the plant material should reach a humidity of 20–30% (Feng et al. 2013).

In the case of raspberry, desiccation of plant explants is performed by placing the capsules in MS medium supplemented with sucrose. The optimal sucrose concentration should be in the range of 0.8–1.0 M, and incubation time on the desiccation

medium can vary from 90 minutes (Gupta & Reed 2006) to as long as 20 hours (Sakai et al. 1990). Desiccation is continued by drying on sterile filter paper and then dehydrating for 6 hours under laminar flow to a moisture content of approximately 20%. After dehydration, the plant material is immersed in liquid nitrogen (Tanaka et al. 2018).

Encapsulation-vitrification

Encapsulation-vitrification is a method that combines two techniques – vitrification and encapsulation-dehydration (Ma et al. 2024). In encapsulation-vitrification, plant explants are encapsulated in alginate, treated with LS, and then dehydrated with a proper vitrification solution (PVS3) before rapid immersion in liquid nitrogen (Condello et al. 2011).

The encapsulation-vitrification approach has been successfully applied to raspberry shoot tips, typically using a stepwise preculture of sucrose followed by dehydration with plant vitrification solutions (Wang et al. 2005). For *Rubus*, PVS2 is the most commonly used vitrification solution. PVS3 [50% (w/v) glycerol + 50% (w/v) sucrose] has also been tested as a DMSO-free alternative, particularly in blackberry protocols (Gupta & Reed 2006; Vujović et al. 2011). Recent droplet-vitrification protocols for raspberry based on PVS2 achieve high survival and regrowth in multiple genotypes with optimized preculture, loading, and duration of PVS2 exposure (Condello et al. 2011; Ma et al. 2024).

Droplet-vitrification

The droplet-vitrification method (droplet freezing) enables ultrarapid cooling and rewarming by placing shoot tips in small droplets of vitrification solution on aluminum foil strips before direct immersion in liquid nitrogen (Condello et al. 2011; Ma et al. 2024). In *Rubus* protocols, shoot tips are typically osmoprotected in LS (e.g., 2 M glycerol, 0.4 M sucrose) for about 20 minutes and then dehydrated with PVS2 (usually for 20–30 minutes, often at 0°C or room temperature, depending on the protocol) before freezing (Gupta & Reed 2006; Condello et al. 2011; Ma et al. 2024).

Rewarming plant explants

After storage in liquid nitrogen (-196°C), shoot tips are rapidly rewarmed to minimize recrystallization, typically by transferring cryovials or foil strips directly to a warm water bath (e.g., 45°C for about 1 minute, followed by a short equilibration at approximately 25°C) (Gupta & Reed 2006). Cryoprotectants are then removed by rinsing or incubating the explants in an unloading or recovery solution with an elevated sucrose concentration (typically 0.75–1.20 M) before

transferring to regeneration medium (Gupta & Reed 2006; Condello et al. 2011; Ma et al. 2024).

Post-culture and the regeneration process

Some authors transfer the rewarmed explants to recovery or regeneration medium and maintain them under standard growth conditions (e.g., in a controlled growth environment, phytotron). Plant recovery is conducted on solid MS regeneration media supplemented with various phytohormones or directly in perlite with 20% water (Table 2).

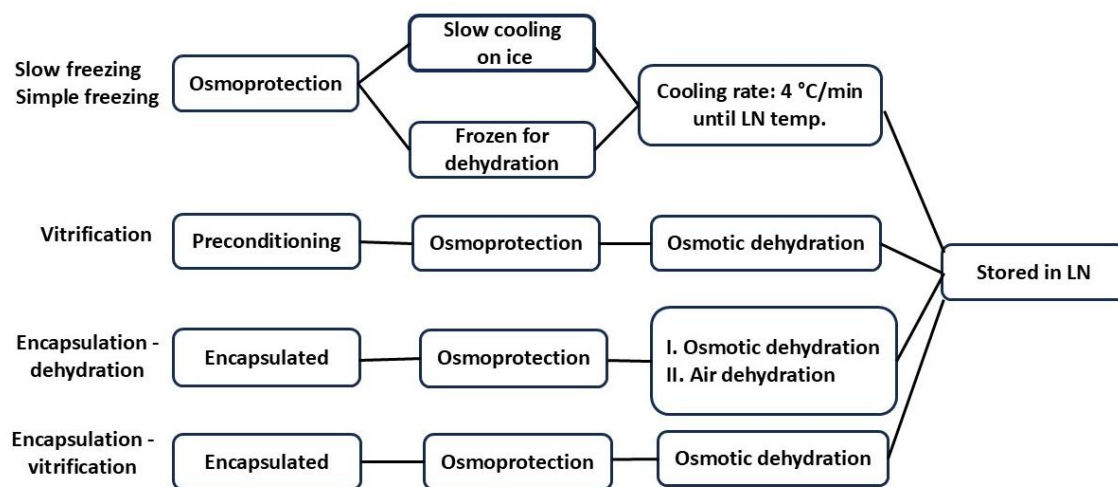


Figure 1. Successive main steps of cryopreservation procedures

Table 1. Characteristics of plant cryopreservation methods

Method	Characteristics	Cryoprotectants	Cooling procedure
Slow freezing	Slow cooling at 0.3–0.5°C per min to -40°C	DMSO, glycerol, ethylene glycol, sucrose	Cooling at 0.3–0.5°C per min to -40°C and then to -196°C
Simple freezing	Freezing at -30°C for dehydration and preservation	DMSO, glycerol, ethylene glycol, sucrose	Freezing at -30°C for dehydration, then cooling to -196°C
Encapsulation-dehydration	Encapsulation in alginate followed by osmotic dehydration	Alginate solution in liquid MS medium, higher sucrose concentrations (0.8–1.0 M); MS medium for dehydration with added sucrose (0.8–1.0 M)	Dehydration followed by rapid immersion in liquid nitrogen
Encapsulation-vitrification	Combination of encapsulation with vitrification for rapid cooling	LS solution (2 M glycerol, 0.4 M sucrose), PVS3	Dehydration with vitrification solution, rapid cooling in liquid nitrogen
Droplet-vitrification	Cryoprotectant droplets rapidly cooled on aluminum foil	LS solution (2 M glycerol, 0.4 M sucrose), PVS2	Cooling plant droplets on aluminum foil and then immersing them in liquid nitrogen
Vitrification	Rapid cooling to form a glassy state without crystallization	Preconditioning: DMSO (5%) for 48 hours, followed by dehydration in LS solution (2 M glycerol, 0.4 M sucrose) Cryoprotectants: PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose), PVS3, or other cryoprotectant combinations (depending on the protocol)	Rapid cooling to -196°C , preventing ice crystals from forming

Table 2. Types and concentrations of phytohormones in media used for raspberry regeneration after cryopreservation

Medium	Phytohormones (mg·L ⁻¹)					Reference
	BA	IBA	IAA	GA ₃	TDZ	
Anderson's	0.5	0.2	0.1	-	-	Reed and Lagerstedt (1987)
MS	1.0	-	-	-	0.2	Vysotskaya et al. (1999)
MS	0	0	0	0	-	Wang et al. (2005)
RUB	2.0	-	-	0.1	-	Gupta and Reed (2006)
NCGR-RUB	2.0	0.1	-	0.1	-	Condello et al. (2011)
SMM	0.5	0.1	-	-	-	Ukhatova et al. (2017)
MS	0.6	0.1	-	-	-	Burian (personal communication, 2021)
MS	0.5	0.1	-	-	-	Ma et al. (2024)
Perlite	-	-	-	-	-	Bobrova et al. (2025)

COMPARISON OF THE EFFECTIVENESS OF RASPBERRY CRYOPRESERVATION

METHODS

In *Rubus*, encapsulation-vitrification and encapsulation-dehydration have been widely applied. For raspberry shoot tips, encapsulation-vitrification provided higher survival and regrowth than encapsulation-dehydration in a direct comparison (Wang et al. 2005). In many *Rubus* species and hybrids, encapsulation-dehydration enabled 60–100% recovery, while vitrification-based methods also provided high recovery for many genotypes (Gupta & Reed 2006).

Both controlled-rate (two-step) cooling and vitrification-based approaches have been used for cryopreservation of *Rubus*, but the rate of regrowth strongly depends on the genotype and the details of the protocol (Wang et al. 2005; Gupta & Reed 2006). Early controlled-rate cooling of raspberry shoot apices was reported by Reed and Lagerstedt (1987). For droplet-vitrification in raspberry, Condello et al. (2011) reported shoot regeneration from 18% of cryopreserved explants. In contrast, a more recent optimization of the protocol increased regrowth to 67–100% in various raspberry genotypes (Ma et al. 2024). Similarly, encapsulation-vitrification provided high average regrowth in many raspberry genotypes (e.g., approximately 68% in 7 genotypes) (Wang et al. 2005).

CONCLUSIONS

Cryopreservation and cryotherapy are effective and proven tools for conserving *Rubus* genetic resources and producing pathogen-free planting material, respectively. In raspberry, shoot-tip cryotherapy relies on the selective survival of meristematic tissues to eliminate viruses and regenerate healthy plants suitable for further propagation (Wang et al. 2008, 2009). Among currently available cryopreservation methods, protocols based on vitrification and encapsulation-dehydration consistently provide high levels of post-thaw survival and plant recovery when key technical parameters, such as explant size, physiological state, preculture conditions, exposure to cryoprotectants, and cooling and rewarming regimes, are appropriately optimized (Wang et al. 2005; Gupta & Reed 2006).

The studies reviewed here demonstrate that cryogenic techniques function as both a long-term conservation strategy and as a practical sanitation method, complementing other *in vitro* techniques. Although the efficiency of the protocol remains genotype-dependent, accumulating experimental evidence confirms that optimized cryopreservation procedures can reliably support the maintenance of healthy raspberry germplasm and the regeneration of viable plants after cryostorage.

Author contribution statement

WK – D – Writing the article

MB – A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article

MOS – A – Research concept and design

DK – C – Data analysis and interpretation

MC – B – Collection and/or assembly of data

Conflicts of Interest

The authors declare no conflict of interest.

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