

Vitrification Cryo-Foil for Apple Cryopreservation and the Seesaw Effect Between Shoot Recovery and Virus Eradication

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Abstract

Improvements of existing cryopreservation protocols are necessary to facilitate long-term preservation of plant germplasm and the cryotherapy-effect of pathogen eradication. This study reported a vitrification (V) cryo-foil/plate methods for cryopreservation of shoot tips and cryotherapy effect in 'Pink Lady' apple. In V cryo-foil/plate protocols, shoot tips were first attached onto aluminum foils/plates using calcium alginate before other procedures. Shoot tips cryopreserved by V cryo-foil required 6.1 weeks to fully recover and 53% of shoot regrowth was obtained, comparable to the Dv cryopreservation. Similar regrowth levels were produced between applying V cryo-foil and Dv cryopreservation to another 4 *Malus* genotypes. Histological observations in shoot tips cryopreserved by Dv and V cryo-foil found only those with surviving apical dome and leaf primordia (LPs) could recover after cryopreservation. In apical meristem of shoot tips cryopreserved by Dv and V cryo-foil, higher surviving probability was detected from the V cryo-foil protocol, and the young LPs showed the highest level of surviving. Virus detection in cryo-derived plants showed apple stem grooving virus and apple chlorotic leaf spot virus were all preserved after cryopreservation, and higher eradication efficiency of apple stem pitting virus (70%) was produced by Dv than the 55% of V cryo-foil. These results supported applying V cryo-foil as an improvement to the widely applied Dv method in shoot tip cryopreservation, and also revealed a seesaw mode between shoot recovery and cryotherapy effect. Once the seesaw moves to increase the recovery after cryopreservation, the cryotherapy-effect on the other side would be decreased.

Key Message

Vitrification cryo-foil cryopreservation resulted in higher viability of apical meristem after cryopreservation, but reduced the cryotherapy effect of virus elimination.

Introduction

Cryopreservation is at present considered the most reliable strategy for long-term preservation of plant genetic resources (Panis 2019; Bettoni et al. 2021; Wang et al. 2021c). Over the past few decades, great efforts have been exerted to develop efficient methods for plant cryopreservation (Wang et al. 2021c). Droplet vitrification (Dv) is easy to perform, and resulted in high post-thaw regrowth levels using shoot tips, and has been shown applicable to a wide range of plant species and genera such as *Allium*, *Citrus*, *Malus*, *Musa* and *Solanum* (Panis 2019; Wang et al. 2018a, 2021c; Panis 2019) as well as recalcitrant and tropical species, which are more sensitive to desiccation (Malik and Chaudhury 2019; Normah et al. 2019). These results confirm that Dv is currently the most widely applicable cryoprotocol for cryopreserving plant germplasm within genebanks, particularly for vegetative propagated crops (Panis et al. 2005; Sakai and Engelmann 2007; Sakai et al. 2008; Wang et al. 2018a, 2021c; Normah et al. 2019; Panis 2019).

When plant tissues are infected with intracellular pathogens such as virus, viroid and phytoplasma, cryopreservation will result in pathogen eradication, due to the killing effects on pathogen-infected cells

(Wang and Valkonen 2009; Zhao et al. 2019). The cryotherapy effect of pathogen eradication has been mostly revealed from shoot tips in which virus-free apical meristem showed higher tolerance towards cryopreservation (Wang and Valkonen 2009; Zhao et al. 2019). The cryotherapy effect has been proved most evident in shoot tips infected by the phloem-limited virus (Bi et al. 2018; Zhao et al. 2019), and was then followed by viruses that cannot infect the apical meristem (Li et al. 2016). As for viruses or viroid that infect the apical meristem, no or very low cryotherapy effect could be obtained, making shoot tip cryopreservation a reliable means for long-term preservation of these pathogens (Wang et al. 2018c; Li et al. 2019).

To facilitate full-scale implementation of plant cryopreservation, as well as the use of cryogenic protocols for pathogen elimination or preservation, further developments of easy-operate and efficient methods are still needed. These efforts would reduce difficulties of technology transfer between laboratories and cryobanks (Bettoni et al. 2021). In the Dv method, shoot tips are treated by a series of solutions including liquid preculture medium, loading, plant vitrification solution (PVS), and unloading solution (Panis et al. 2005; Wang et al. 2021c). These treatments require frequent changes of solutions using pipettes, which may cause damage to and loss of shoot tips (Wang et al. 2021c). The Dv method also requires a precise control of the duration of exposure to PVS, particularly when performed at room temperature (Panis et al. 2005; Wang et al. 2021c). The recently developed vitrification cryo-plate (V cryo-plate) method adheres shoot tips on aluminum cryo-plates by calcium-alginate before direct immersion in treatment in liquid media (Yamamoto et al. 2011, 2012; Niino et al. 2019). Samples can be easily handled, thus reducing risks of damaging or losing the explants following cryoprotocols (Yamamoto et al. 2012; Bettoni et al. 2019b; Niino et al. 2019). However, the use of cryo-plates requires purchase of manufactured plates, which may be unavailable, particularly in developing countries. Therefore, the use of aluminum foil, which is more readily available as a replacement of cryo-plates, should be tested.

The present study attempted to establish a vitrification cryo-foil (V cryo-foil) method, based on the Dv method previously reported for cryopreservation of apple by our group (Li et al. 2015). Then, we compared shoot regrowth, surviving patterns and virus eradication between the V cryo-foil and Dv methods in apple. Virus immunolocalization in cryopreserved shoot tips were used to verify the cryotherapy-effect of V cryo-foil and Dv.

Materials And Methods

Plant materials

In vitro stock shoots of apple cv. 'Pink Lady' (*Malus domestica*) were used for optimizing the V cryo-plate and V cryo-foil protocols for shoot tip cryopreservation, histological study and evaluations of cryotherapy effect for virus eradication. 'Pink Lady' was infected with apple stem grooving virus (ASGV), apple stem pitting virus (ASPV) and apple chlorotic leaf spot virus (ACLSV), as confirmed by reverse transcription-polymerase chain reaction (RT-PCR). The *in vitro* virus-free apple cv. 'Yanfu-8' (*M. domestica*), wild species Qiuzi (*M. prunifolia*) and two rootstocks 'M9' (*M. paradisiaca*) and 'M26' (*M. pumila*) were used to

test the applicability of the V cryo-foil method established in the present study. *In vitro* stock shoots were maintained on shoot maintenance medium (SMM) composed of Murashige and Skoog (1962) medium (MS, M519, PhytoTech Labs) supplemented with sucrose at 30 g.L⁻¹, 6-benzyladenine (Sigma) at 0.6 mg.L⁻¹, indole-3-butyric acid (Sigma) at 0.1 mg.L⁻¹. The pH of the medium was adjusted to 5.8, prior to autoclaving at 121°C for 20 min. The stock cultures were kept constantly at 24 °C under a 16-h photoperiod provided by cool-white, fluorescent tubes set at a light intensity of 50 μmol.m⁻².s⁻¹. Subculture was performed every 4 weeks.

Dv cryopreservation

Dv cryopreservation was performed as reported by Li et al. (2015). The pH of all the media and solutions used in cryopreservation were adjusted to 5.8 prior to autoclaving. Briefly, shoot tips about 2.0 mm in length with 4-5 leaf primordia (LPs) were excised from 4-week-old *in vitro* stock cultures and incubated in SMM overnight. Shoot tips were then transferred to a loading solution (MS medium supplemented with 2 M glycerol and 0.8 M sucrose) for 24 h before the dehydration by plant vitrification solution 2 (PVS2) (Sakai et al. 1990) for 0-80 min at 24 °C. After PVS2 dehydration, shoot tips were transferred into PVS2 droplets on aluminum foil (about 3 × 0.9 cm, Fig. 1A) before direct immersion in liquid nitrogen (LN). After immersing in LN for 1 h, the aluminum foil, on which ten shoot tips were attached, was transferred rapidly into unloading solution (1.2 M sucrose in MS) for thawing, and incubated for 20 min at 24°C. Shoot tips were then post-thaw cultured in SMM for recovery. Post-thaw culture was performed in the dark for the first 3 days, and was then under weak light conditions (10 μmol.m⁻².s⁻¹) for the next 4 days before moving to the same conditions as for the *in vitro* stock shoots. Subculture was performed when light conditions were changed and was also done every 4 weeks at normal light conditions. Shoot regrowth was confirmed when shoot tips developed into normal shoots (≥ 5.0 mm in length) with a new leaf emerging after 8 weeks of postculture on SMM. We recorded the weeks of post-thaw culture required for confirming the full recovery of cryopreserved shoot tips.

V cryo-plate and V cryo-foil cryopreservation

The V cryo-plate and V cryo-foil protocols for shoot tip cryopreservation were established based on the Dv protocol developed by Li et al. (2015). Briefly, shoot tips were excised and incubated overnight in SMM as the Dv method. The attachment of shoot tip using calcium-alginate was performed according to Feng et al. (2013) with modifications. In the Day 2, shoot tips were transferred into liquid MS medium containing 2.5 % (w/v) Na-alginate (A0682, Sigma), 2 M glycerol and 0.4 M sucrose. After 10 minutes of incubation, shoot tips were transferred to the wells of cryo-plates (about 25 × 9 × 0.5 mm) and the droplets on the foils, both containing 5 μl of Na-alginate solutions for the V cryo-plate and V cryo-foil protocols (Fig. 1B and C). Then, calcium chloride solution (liquid MS medium containing 0.1 M calcium chloride, 2 M glycerol and 0.4 M sucrose) was gently poured (200 μl/foil or plate) to cover all the areas of plate/foil on which shoot tips were attached. After 15 minutes of incubation, the excessive calcium chloride solution was removed gently by filter paper and cryo-plates and cryo-foils attached with shoot tips (Fig. 1B and C) were incubated by loading solution as the Dv method for 24 hours at room temperature. The dehydration

by PVS2, immersion in LN, thawing in unloading solution, post-thaw cultures and shoot regrowth evaluations were performed as the Dv method. Comparison of major steps between Dv and V cryo-foil methods were illustrated in Fig. 1D.

Evaluation of shoot tip survival and histological study

Comparison of shoot tip surviving was made between the conventional Dv with 40 minutes of PVS2 exposure and newly optimized V cryo-foil protocols with 50 minutes of PVS2 treatment. Histological study was conducted in cryopreserved shoot tips 3 and 7 days after post-thaw culture, according to the method reported by Feng et al. (2013), to further study the surviving patterns of shoot tips following cryopreservation. Shoot tip right after cryopreservation and after one-day of post-thaw culture were also observed, and not used due to the undistinguishable features between the surviving and the dead tissue (Data not shown). In brief, shoot tips were fixed with formalin-acetic acid-alcohol solution, dehydrated in an incremental ethanol series before xylene infiltration. Then samples were infiltrated and embedded by paraffin. Sections (7 µm thick) were obtained by a rotary microtome (RM 2235, Leica, Nussloch, Germany) and stained with 0.1% toluidine blue. The samples were observed with a light microscope (DM 2000, Leica, Wetzlar, Germany). Excised shoot tips cultured directly on SMM for 3 days were served as the positive control (survived cells), while negative control (killed cells) was obtained from those excised, directly immersed in LN and post-thaw cultured for 3 days. Both positive and negative controls were fixed, sectioned, stained and observed as described above.

The cryopreserved samples were compared between Dv and V cryo-foil, according to three types of surviving patterns, i.e., surviving of both apical dome (AD) and LP, only surviving of LP and none surviving samples. In addition, images representing 10 randomly selected shoot tips of each treatment were illustrated with Adobe Illustrator CC 2019 using red color and green color to indicate the killed and survived tissues, respectively (Fig. 4a, b). After lowering the transparency of the illustrated objects, images were stacked and merged using Adobe Photoshop CC 2019, to demonstrate the probabilities of survival in different areas of cryopreserved shoot tips, based on different colors and hues. These illustrations were made for comparing the surviving pattern between Dv and V cryo-foil after 3 and 7 days of postcultures.

Virus detection and assessment of virus eradication efficiency

In virus detection from the *in vitro* stock cultures, total RNA was extracted from fresh shoots (0.1 g) using a Spectrum Plant Total RNA Kit (Sigma) following the manufacturer's instructions. cDNA was synthesized from 2 µg total RNA using inNova Uscrip II All in One First-Strand SuperMix (AR121-Mix, Innovagene Biotech, Changsha, China), according to the manufacturer's instructions. The PCR was performed in 25 µL reaction volume containing 12.5 µL of 2 × Taq PCR PreMix (GS101, Innovagene Biotech, Changsha, China), 1 µL of each primer at 10 µM, 2 µL of cDNA and 8.5 µL of Rnase-free water. The forward primer (5'-ATGTCTGGAACCTCATGCTGCAA-3') and the reverse primer (5'-TTGGGATCAACTTTACTAAAAGCATAA-3') were used for apple stem pitting virus (ASPV) to amplify a product of 370 bp (Menzel et al. 2002). The forward primer (5-CTGCAAGACCGCGACCAAGTTT-3') and the

reverse primer (5'-CCCGCTGTTGGATTTGATACACCTC-3') were used for detection of apple stem grooving virus (ASGV), amplifying a product of 524 bp (MacKenzie et al. 1997). In detection of apple chlorotic leaf spot virus (ACLSV), the forward primer (5'-TTCATGGAAAGACAGGGGCAA-3') and the reverse primer (5'-AAGTCTACAGGCTATTTATTATAAGACTAA-3') were used according to Menzel et al. (2002) to produce a product of 677 bp. The results from detection of other major viruses and viroid that prevail in China are not provided in this study. PCR amplification was performed in a thermal cycler (Mastercycler, Eppendorf, Germany) using 94 °C for 5 min, and was followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis in a 1.5% agarose (w/v) in tris-acetate buffer with 0.01% DuRed Gel Stain (v/v, A168, Biomiga, China), and visualized under ultraviolet light. In plants regenerated after cryopreservation, virus detection was performed *in vitro* after 3 and 4 months of post-thaw cultures. Those that were ASPV-free were transferred to soil and grown in greenhouse conditions for 9 months, encompassing one dormant season (from August 2020 to May 2021) for final evaluation of their ASPV-free status.

Virus localization

To study the virus distribution in the shoot tips before and after cryotherapy, ASPV and ASGV were localized in shoot tips excised from *in vitro* apple cv. 'Pink Lady' and in cryopreserved shoot tips after 3 days of post-thaw culture, according to Wanget al. (2008). Samples were fixed, dehydrated, paraffin-embedded and sectioned as the histological observation of the present study. Then sections on slides were washed twice in xylene to remove paraffin, rehydrated, and treated with phosphate-buffered saline (PBS). Samples were blocked with PBS containing 4% (w/v) bovine serum albumin before overnight incubation with ASPV monoclonal and ASGV polyclonal antibodies (Bioreba; dilution 1:500 in PBS) to the virus coat protein at 5 °C. Samples were then washed three times with PBS and incubated with anti-rabbit and anti-mouse antibodies (Sigma; dilution 1:400 in PBS) for 20 minutes at room temperature in localization of ASGV and ASPV, respectively. After three rinses with PBS, sample were stained with freshly prepared fuchsin substrate solution (based on New Fuchsin; Sigma), and observed under a light microscope (DM 2000; Leica) for detection of purple staining that indicated the presence of virus. In shoot tips cryopreserved by V cryo-foil and Dv, respectively, ten samples that showed surviving of AD and LP were randomly selected and applied in virus localization of ASGV and ASPV. Surviving tissues and dead tissues were illustrated as histological study, and blue color was used to denote the virus-infected tissues as stained from the virus localization.

Comparison of genotype responses between Dv and V cryo-foil

The V cryo-foil method optimized above was tested for its applicability to additional four virus-free apple genotypes including cv. 'Yanfu-8', two rootstocks 'M26' and 'M9', and one wild species 'Qiuzi'. The Dv method with 40 minutes of PVS2 incubation was applied in comparison with the optimized V cryo-foil method.

Experimental design and data statistical analysis

In the experiment regarding shoot tip cryopreservation, 10 shoot tips were used in each treatment of 3 replicates and the whole experiment was repeated twice. Least significant differences were calculated at $p < 0.05$ by student's t test or one-way analysis of variance with Tukey's test, depending on the number of treatments. Twenty cryo-derived plants were randomly selected from the Dv and V cryo-foil treatments in virus detection. As for histological observation of surviving tissues after cryopreservation, 10 shoot tips were used in each treatment of three replicates. In virus localization, 10 shoot tips were randomly selected before cryopreservation, and from those showed surviving tissues in both AD + LP after cryopreservation, respectively, and used for analysis.

Results

Shoot tip regrowth after cryopreservation by Dv, V cryo-plate and V cryo-foil in 'Pink Lady'

As exposure to PVS2 at room temperature was suitable for cryopreservation of *Malus* shoot tips (Li et al. 2015), the duration effect of the PVS2 treatment was tested at room temperature in this study. Shoot tip regrowth after cryopreservation significantly increased in all the tested protocols when duration of PVS2 exposure extended from 0 to 40 min (Fig. 2a). However, no significant variation was detected between different protocols when same duration of PVS2 exposure was applied (Fig. 2a). The highest regrowth level (48%) was produced after 40 minutes of PVS2 exposure in Dv protocol, while 50 minutes of PVS2 treatment resulted in the highest regrowth levels in both V cryo-foil and V cryo-plate protocols (Fig. 2a). Noticeably, the highest regrowth level was produced in V cryo-foil protocol as 53% of shoot regrowth was produced, but no significant variation was produced from 40-60 minutes of PVS2 exposure. Of the 20 shoot tips that showed direct regrowth by 8 weeks of post-thaw culture after cryopreservation, 6.1 weeks was required on average for shoot recovery to be visually confirmed in the V cryo-foil protocol, shorter than the 6.6 weeks taken after the Dv treatment (Fig. 2b). Since comparable results were produced from three tested protocols, the new V cryo-foil method was compared with the traditional Dv method in terms of the genotype response, histological study and cryo-therapy effect of virus eradication.

Testing of genotype response to V cryo-foil method

When the optimized V cryo-foil method was tested to other four *Malus* genotypes, shoot tip regrowth of 50%, 53% and 30% were obtained from 'Yanfu-8', 'M9' and 'Qiuzi', respectively, and were higher than regrowth levels produced by Dv protocol (Table 1). Lower regrowth level of 43% was produced by V cryo-foil in rootstock 'M26', as compared with the 46% obtained from the Dv method (Table 2). No significant difference was detected by Student's t -test at $p < 0.05$ in regrowth levels between V cryo-foil and Dv protocol for each *Malus* genotype.

Histological study of shoot tips cryopreserved by Dv and V cryo-foil

In histological study of freshly excised shoot tips (positive control), cells were densely stained in cytoplasm and showed well-preserved structures (Fig. 3a, b). In contrast, disrupted cell structures, lightly stained cytoplasm and shrunken nucleus were characteristics of killed cells from the negative control

(Fig. 3c, d). In shoot tips post-thaw cultured 3 days after cryopreservation (3 DAC), three types of surviving patterns were observed and categorized as shoot tips showed surviving AD and LP (Fig. 3e), shoot tips with only surviving LP (Fig. 3f) and none surviving shoot tips (Fig. 3g). In shoot tips cryopreserved by Dv protocol, 44% of shoot tips showed well-preserved AD and LP, while 28% of shoot tips showed survived LP, same to the fully killed shoot tips (Table 2). After V cryo-foil cryopreservation, 52%, 26% and 22% of shoot tips showed surviving AD and LP, only surviving LP and none surviving, respectively. No significant difference in the percentage of surviving pattern was detected between Dv and V cryo-foil protocols, as well as between shoot tips observed at 3 and 7 DAC (Table 2). Different surviving patterns could be visually detected at 14 DAC (Fig. 3h-j).

Probabilities of cell surviving in shoot tips cryopreserved by Dv and V cryo-foil

After overlaying the red-green images obtained from histological observation, greener colors were presented when surviving tissues of different samples were overlaid, while darker red was produced from the overlaying of dead tissues (Fig. 4c-f). In the areas where both dead and survived samples stacked, yellowish color was produced (Fig. 4c-f). In shoot tips cryopreserved by Dv, highest level of green color was illustrated on the young LPs at 3 DAC, indicating the highest surviving probability was from young LPs after cryopreservation (Fig. 4c). Similar results were produced in the young LPs of shoot tips cryopreserved by the V cryo-foil (Fig. 4e). The AD of shoot tips was illustrated with a more yellowish green from both Dv and V cryo-foil treatments, as only about half of cryopreserved shoot tips showed viability in the AD, (Fig. 4c, e). Noticeably, the tissues below the AD still kept yellowish green in shoot tips cryopreserved by V cryo-foil (Fig. 4e, f), and were larger than those treated by Dv at both 3 and 7 DAC (Fig. 4c, d). The darkest red was illustrated from lower parts of the shoot tips at both 3 and 7 DAC, regardless of the cryopreservation methods (Fig. 4c-f).

Cryotherapy-effect of virus eradication in 'Pink Lady' recovered after cryopreservation

Analysis using RT-PCR for virus detection produced specific bands of 524 bp, 677 bp and 370 bp for ASGV, ACLSV and ASPV, respectively, from the positive controls and 'Pink Lady' before cryopreservation, whereas no such bands were produced from the negative controls (Fig. 5a-c). After shoot tip cryopreservation, Dv and V cryo-foil methods failed to produce any recovered plant that was free from ASGV and ACLSV (Table 3, Fig. 5a, b), while ASPV-free shoots were obtained from both treatments (Fig. 5c). Lower level of ASPV eradication (55%) was produced by V cryo-foil method as compared with the 70% produced after the Dv treatment (Table 3). The infections of ASGV and ACLSV were confirmed in all the cryo-recovered shoots after two rounds of *in vitro* detection. No difference was produced in the ASPV-free status between the second *in vitro* and the final detections at greenhouse.

Virus localization in shoot tips before and after V cryo-foil

Immunohistological staining of virus resulted in purple colors in shoot tips of positive controls for localization of ASGV and ASPV (Fig. 6a and b), whereas no such color was detected from the virus-free negative controls (Fig. 6c, d). In shoot tips before cryopreservation, purple staining of ASGV was clearly

seen from the apical meristem, leaf primordia, and the lower parts of the shoot tip (Fig. 6e, e1), while ASPV could only be detected from the lower parts of the shoot tip (Fig. 6f, f1). After the shoot tip cryopreservation, ASGV could still be clearly detected from all the ADs and LPs of survived shoot tips after 7 days of post-thaw culture (Fig. 6g). The level of ASPV staining was greatly reduced due to the killing effects of cryopreservation on tissues from the lower part of the shoot tip. Five and 3 samples after the V cryo-foil and Dv methods, respectively, showed faint staining of ASPV in the lower part of surviving tissues or old LPs (Fig. 6h), while no viral signals could be detected from the other cryopreserved shoot tips in ASPV localization (Fig. 6i).

Discussion

This study reported a V cryo-foil/plate method for cryopreservation of apple *in vitro* shoot tips. Shoot regrowth levels produced by the V cryo-foil/plate method were similar to those by the Dv in cryopreserved shoot tips of cv. 'Pink Lady'. The V cryo-foil resulted in 30%-53% of shoot regrowth levels across the other 4 *Malus* species, similar to 23%-46% obtained after the Dv protocol. Histological observation of surviving tissues found higher level of surviving probability in shoot tips after the V cryo-foil. In plants recovered after cryopreservation, the V cryo-foil produced 55% of ASPV-free plantlets, lower than the 70% produced by the Dv. Both V cryo-foil and Dv failed to eradicate ACLSV and ASGV.

When performing V cryo-plate/foil methods for cryopreservation, shoot tips are firstly attached onto plates/foils before subsequent loading and PVS treatment (Yamamoto et al. 2012, 2011). In an encapsulated protocol for cryopreservation of apple, Feng et al. (2013) found the viability of apple shoot tips was maintained after encapsulating with 2 M of glycerol followed by 5 days of preculture at 0.5 M of sucrose. We therefore applied the alginate gel with 2 M of glycerol and 0.4 M of sucrose, lowered than the 2 M of glycerol and 0.8 M of sucrose of the subsequent preculture/loading step, for attaching shoot tips on foils. In developing the V cryo-plate protocol for cryopreservation of mint and mulberry, Yamamoto et al. (2012) and Tanaka et al. (2019) showed respectively that the regrowth level was not affected with the addition of sucrose (at 0.4 M) in alginate gel. The present study did not optimize the solutes of the alginate gel, as it may have quite limited influence on the regrowth of cryopreserved shoot tips, as suggested by Yamamoto et al. (2012) and Tanaka et al. (2019).

In vitrification-based methods for plant cryopreservation, the extent of PVS infiltration is the most key factor affecting the recovery of tissues following cryopreservation (Sakai et al. 1990; Yamamoto et al. 2012; Li et al. 2015; Wang et al. 2021c). In comparison with the traditional Dv method, it has been found longer PVS2 exposure is required in V cryo-plate/foil methods for the optimized cryopreservation. The gel encapsulated around the tissues may slow the penetration of PVS as proposed by Wang et al. (2021c), and led to the longer PVS2 incubation in V cryo-plate/foil methods of present study. This study also found, as compared with Dv method, comparable regrowth levels were obtained from V cryo-foil in 'Pink Lady' and another 4 tested genotypes, and similar post-thaw culture periods were required to confirm the shoot regrowth. Studies on comparing the newly developed V cryo-plate with conventional Dv are quite limited. Bettoni et al. (2019b) found that the V cryo-plate protocol resulted in 68-70% of shoot regrowth,

comparable to 53-70% of the Dv method in cryopreservation of *Vitis* shoot tips. Although no significantly improved regrowth was produced by V cryo-plate methods of Bettoni et al. (2019b) and the V cryo-foil of present study, all these results supported the use of V cryo-plate/foil methods as an improvement for conventional Dv method in plant cryopreservation, due to the easy handling of the procedures.

In shoot tips after cryopreservation, three types of surviving patterns were visually detected in this study after 2 weeks of post-thaw culture. Similar recovery patterns were reported by Li et al. (2015) as callus without shoot regrowth, leaf formation without shoot regrowth and normal shoot regrowth in cryopreserved apple cv. 'Gala' shoot tips. Histological observation of this study found about 50% of cryopreserved shoot tips showed clear surviving tissues from the apical meristem and young leaf primordia. These results were similar to the 48% and 53% of regrowth levels obtained from the optimized Dv and V cryo-foil protocol, respectively, indicating the cryo-recovered plants of present study derived from shoot tips with surviving AD and LPs. The direct regrowth observed from the present study would ensure the genetic stability of cryo-recovered plants, as indirect regrowth through callus may result in somaclonal variation (Miguel and Marum, 2011; Wang et al., 2021a).

It has been recognized when cells of shoot tips are exposed to cryopreservation, those from the AD and youngest LPs are more likely to survive, due to their small vacuole size and low free water content (Wang et al. 2008; Mathew et al. 2018; Bettoni et al. 2019a; Wang et al. 2021b). Similar patterns were presented in the tissues under the AD of this study as the surviving probabilities decreased further away from the AD of shoot tips cryopreserved by both Dv and V cryo-foil. Noticeably, higher surviving probability was detected from overlaid samples cryopreserved by V cryo-foil. The improved surviving from V cryo-foil may be a consequence of the less mechanical damage achieved by avoiding transferring the PVS-stressed shoot tips prior to freezing, but not at the level to significantly improve the shoot regrowth. To the best of our knowledge, this is the first study on histological observation in cryopreserved shoot tips between Dv and V cryo-plate/foil methods.

The histological evaluation of present study also showed higher surviving probability in some tissues of younger LPs, as about 20% of cryopreserved shoot tips only showed surviving LPs after the Dv and V cryo-foil methods. Similar pattern was also observed as the 'leaf formation without shoot regrowth' by Li et al. (2015) in apple 'Gala' after shoot tip cryopreservation. The strong tolerance of LP tissues towards cryopreservation was also noted earlier by Grout and Henshaw (1980) and Fukai and Oe (1990), as shoot regrowth after cryopreservation was mainly from the LP of potato and chrysanthemum, respectively. Although these earlier studies applied mainly DMSO as the cryoprotectant and produced unsatisfied results, all these results did suggest that in more severe conditions, more differentiated cells in youngest LPs may reflect higher resistance, rendering cells of AD not the most tolerant towards cryopreservation. As cryopreservation procedures also resulted in an outburst of reactive oxygen species in plant tissues (Chen et al. 2015; Ren et al. 2021), one of the reasons for lowered tolerance in cells of AD might be the lack of functional chloroplasts, which regulated the plant responses to abiotic and biotic stress conditions (Mamaeva et al. 2020). Nevertheless, in addition to the free water content which is distinguishable by the vacuole size that affected the 'live or die' of cells after cryopreservation, the ability

of cells in response to osmotic stress should also be considered and investigated for better insight of shoot tip recovery after cryopreservation.

In shoot tips recovered after cryopreservation, intracellular parasites such as virus could either be eradicated or preserved for different studies and uses (Zhao et al. 2019). The former situation is referred as the cryotherapy effect (Wang and Valkonen 2009). In the present study, the cryotherapy effect of Dv and V cryo-foil were evaluated in 'Pink Lady' infected with three viruses. We showed that ASGV and ACLSV were preserved in all the cryo-recovered shoots, while ASPV could be eradicated after cryopreservation. Similar results were also reported by Li et al. (2016) for eradication of ASGV and ASPV from apple rootstocks 'M9' and 'M26'. The ASGV localization of present study and Li et al. (2016) both showed distribution of ASGV within the AD and young LPs before and after cryopreservation, while ASPV was detected from the tissue with low surviving probability after cryopreservation, such as the lower part of the shoot tips and older LPs. These results well explained the different cryotherapy effects in elimination of ASGV and ASPV from apple. The present study also found higher frequency of ASPV eradication was produced from the Dv method, making V cryo-foil more applicable for preserving this virus. The lower frequency of ASPV eradication from V cryo-foil also indicated higher number of viable cells were in shoot tips cryopreserved by this method. Since almost all the cryotherapy studies applied optimized cryopreservation protocols (Zhao et al. 2019), an improved virus eradication could be obtained by lowering the regrowth level after shoot tip cryotherapy, as suggested by Wang et al. (2013), Bettoni et al. (2021) and Wang et al. (2021b). Zhao et al. (2018) and Liu et al. (2021) reported that lowering the shoot tip regrowth by applying thermotherapy prior to cryotherapy improved virus eradication in apple. Summarizing all the results reported previously and from the present study, a seesaw model is proposed between the high level of shoot regrowth and the cryotherapy-effect of pathogen eradication (Fig. 7). On one side, fewer pathogen infection (Wang et al. 2018b), cold-acclimation (Niino et al. 1992), and improved cryopreservation protocol (the present study) would contribute to improved shoot recovery after cryopreservation, particularly in cryotolerant species (Wang et al. 2021b), but minimize the cryotherapy effect of pathogen eradication. Of the other side, pathogen infection, thermotherapy and not optimized cryopreservation protocols may result in lowered shoot tip regrowth, evidencing the cryotherapy effect of pathogen eradication.

In conclusion, the present study highlights the establishment of V cryo-foil method based on traditional Dv method for cryopreserving apple shoot tips. Histological study and evaluation on virus elimination showed, as compared with the traditional Dv method, higher level of viability could be obtained in the apical meristem of shoot tips cryopreserved by V cryo-foil protocol, thus decreasing the cryotherapy-effect of ASPV eradication. The cryo-foil method is more readily available than protocols using cryo-plates, making it a promising alternative for cryo-plate based protocols. The V cryo-foil protocol could be applied as an improvement to the widely applied Dv method in cryobanking of plant genetic materials, but not used alone for efficient virus eradication from apple.

Declarations

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Authorship contribution statement

Min-Rui Wang and **Jun-Hua Bao** conducted cryopreservation experiments, data collection and histological study. **Xiao-Yan Ma**, **Ling-Ling Xie** and **Li-Ying Zhu** assisted in histological study and performed virus detection and localization. **Xian Lu** and **Mei-Zi Liu** maintained *in vitro* tissue cultures and greenhouse cultured plants. **Min-Rui Wang** prepared the manuscript draft. **Dong Zhang** and **Qiao-Chun Wang**: Conceptualization, Funding acquisition, Supervision and Reviewing the manuscript.

Conflict of interest The authors declare that they have no competing conflict of interest.

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Tables

Table 1 Regrowth of shoot tips of various apple genotypes cryopreserved by vitrification cryo-foil and droplet vitrification

| Genotypes | Regrowth after cryopreservation by | |
|------------------------------------|------------------------------------|-----------------------|
| | Vitrification cryo-foil | Droplet vitrification |
| Yanfu-8 (<i>Malus domestica</i>) | 50 ± 5 | 40 ± 8 |
| M9 (<i>M. paradisiaca</i>) | 53 ± 5 | 43 ± 5 |
| M26 (<i>M. pumila</i>) | 43 ± 3 | 46 ± 3 |
| Qiuzi (<i>M. prunifolia</i>) | 30 ± 5 | 23 ± 3 |

Data are presented as mean ± SE, and no significant difference was detected by Student's *t*-test at $p < 0.05$ in regrowth levels between V cryo-foil and DV protocol.

Table 2 Percentages of surviving patterns in apple 'Pink Lady' shoot tips cryopreserved by droplet-vitrification and vitrification cryo-foil protocols.

| Days after cryopreservation ^a | Percentage of surviving patterns ^b | | | | | |
|---|---|-------------|--------------|-------------|----------------|-------------|
| | Surviving AD and LP | | Surviving LP | | None surviving | |
| | Dv | V cryo-foil | Dv | V cryo-foil | Dv | V cryo-foil |
| 3 | 44 ± 2 ^c | 52 ± 5 | 28 ± 3 | 26 ± 3 | 28 ± 5 | 22 ± 5 |
| 7 | 43 ± 3 | 53 ± 3 | 23 ± 3 | 20 ± 5 | 33 ± 5 | 26 ± 3 |

Data are presented as means ± SE.

^aHistological observation was made to verify the surviving pattern of shoot tips at 3 and 7 days after cryopreservation, while visual observation was applied after 14 days of postculture.

^bThree types of surviving patterns were observed and clarified as shoot tips with viable apical dome and leaf primordia (Surviving AD+LP), with only surviving leaf primordia (Surviving LP) and totally killed shoot tips (None surviving).

^cNo significant difference was detected by Student's *t*-test at $p < 0.05$ in the percentage of surviving patterns between V cryo-foil and DV protocol, as well as between shoot tips 3 and 7 days after cryopreservation.

Abbreviations: Dv, droplet vitrification; V cryo-foil, vitrification cryo-foil; SE, standard error.

Table 3 Effects droplet-vitrification and vitrification cryo-foil methods on elimination of apple stem grooving virus, apple chlorotic leaf spot virus and apple stem pitting virus from apple cv. 'Pink Lady' plants

| Cryopreservation protocol | Virus eradication efficiency (%) ^a | | |
|---------------------------|---|----------|------------|
| | ASGV | ACLSV | ASPV |
| DV | 0 (0/20) | 0 (0/20) | 70 (14/20) |
| V cryo-foil | 0 (0/20) | 0 (0/20) | 55 (11/20) |

^aNumbers in parentheses are the number of samples showing a negative reaction/total sample tested by reverse transcription-PCR.

Abbreviations: ACLSV, apple chlorotic leaf spot virus; ASGV, apple stem grooving virus; ASPV, apple stem pitting virus; DV, droplet-vitrification; V cryo-foil, vitrification cryo-foil.

Figures

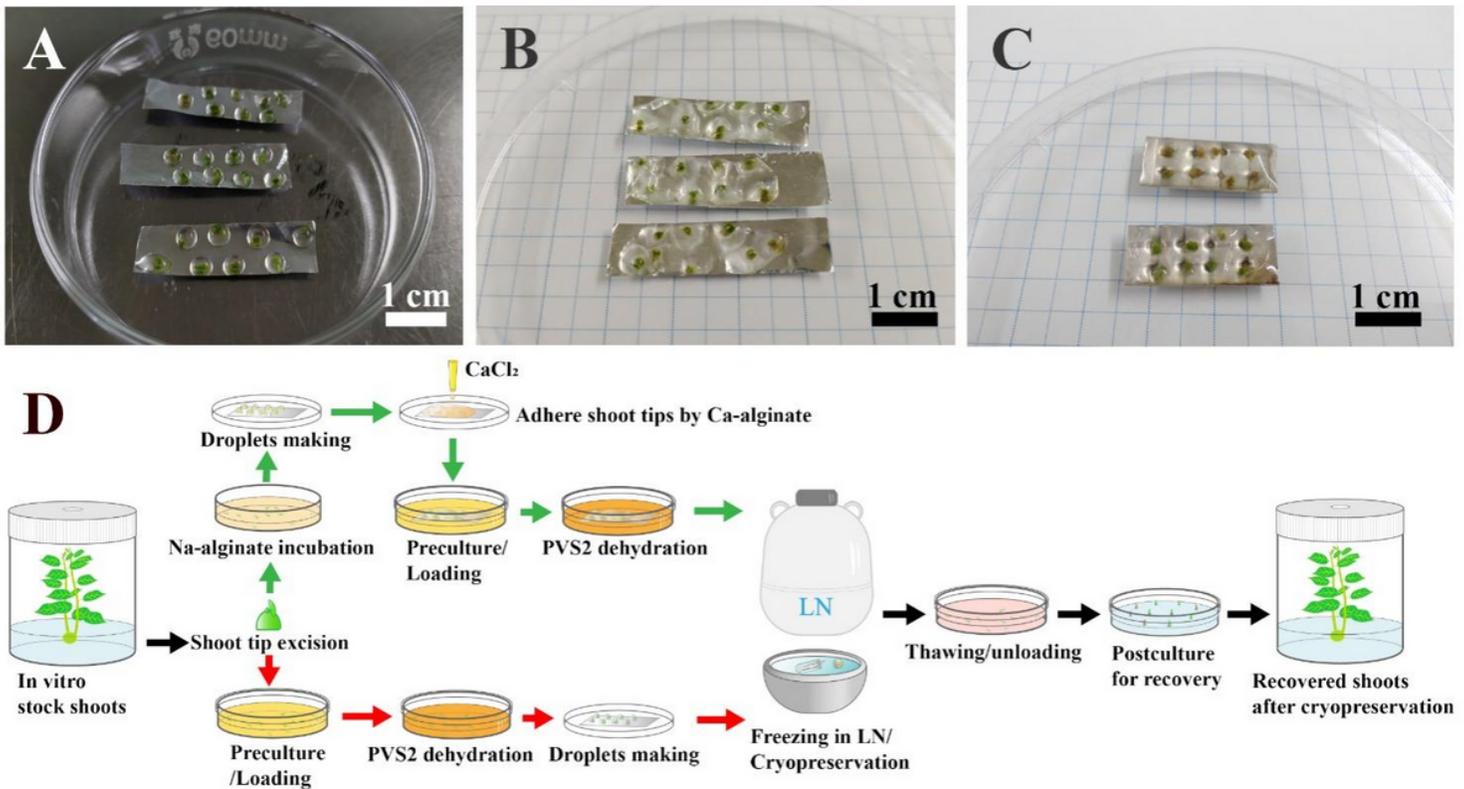


Figure 1

Shoot tip cryopreservation of apple cv. 'Pink Lady' by droplet-vitrification (Dv), vitrification cryo-plate (V cryo-plate) and vitrification cryo-foil (V cryo-foil). **A**, an illustration of droplets making in Dv method. **B** and **C** are illustrations of adhering shoot tips to foils (**B**) and plates (**C**) by Ca-alginate in V cryo-foil and V cryo-plate method, respectively. **D**, schematic illustration of V cryo-foil (green arrows) and Dv (red arrows).

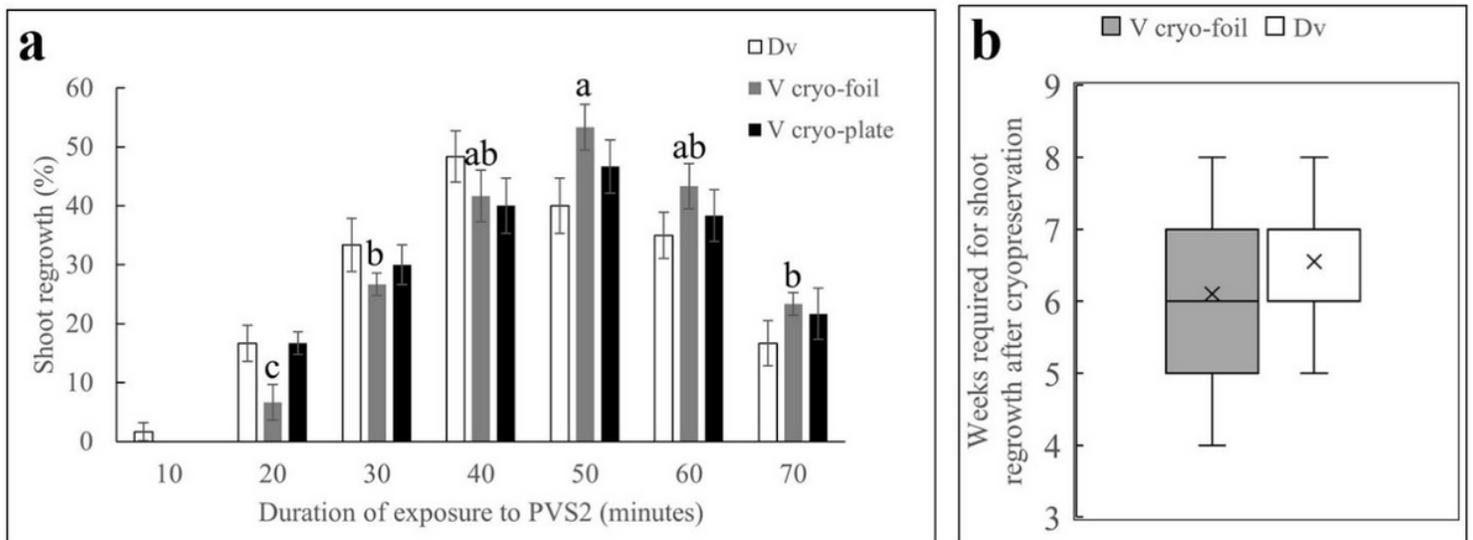


Figure 2

Evaluations on the shoot regrowth after cryopreservation. **a**, effects of PVS2 exposure on shoot regrowth of cryopreserved apple cv. 'Pink Lady' after droplet vitrification (Dv), vitrification cryo-foil (V cryo-foil) and vitrification cryo-plate (V cryo-plate methods); **b**, number of weeks required for confirming the post-thaw regrowth after the V cryo-foil and Dv method. Data of **a** are presented as mean \pm SE. Different letters from the V cryo-foil treatments were indicating significant difference detected by one-way analysis of variance with Tukey's test at $p < 0.05$. No significance was detected from shoot regrowth between different methods when same duration of PVS2 exposure was applied in **a**. The 'x' of **b** indicate the average value of each treatment and no significant difference was detected with student's *t* test at $p < 0.05$ (n=20).

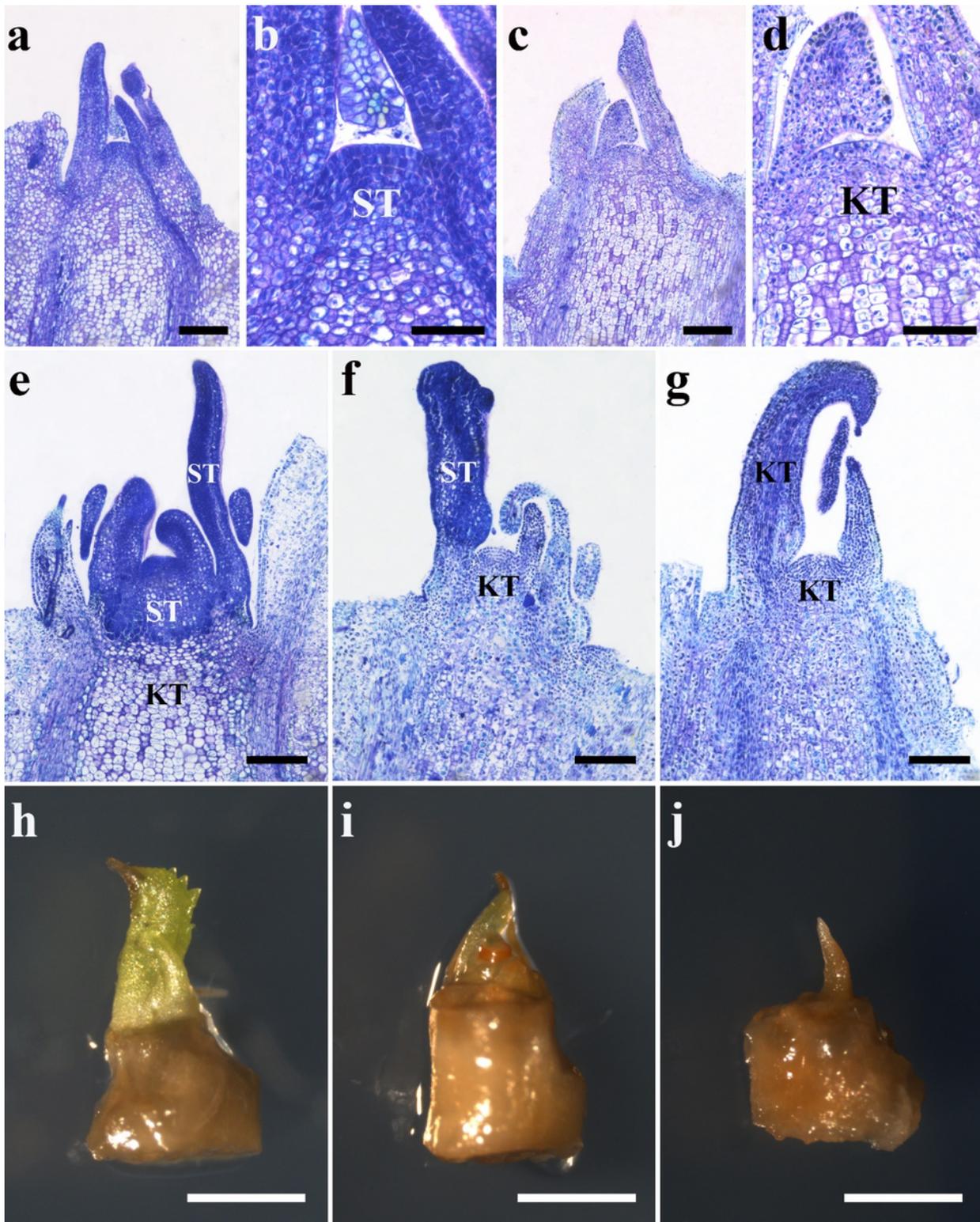


Figure 3

Histological observations of surviving tissues in cryo-treated shoot tips after vitrification cryo-foil (V cryo-foil) cryopreservation in 'Pink Lady' apple. **a**, 'Pink Lady' shoot tip freshly excised and cultured for 3 days, serving as the positive control for living tissues. **b**, a closer view of apical meristem region in the shoot tip of 'a'. **c**, 'Pink Lady' shoot tip freshly excised, immediately immersed in LN and post-thaw cultured for 3 days, serving as the negative control for killed tissues. **d**, a closer view of apical meristem region in 'c'. **e**, **f**

and **g** are shoot tips cryopreserved by V cryo-foil protocol and post-thaw cultured for 3 days, showing surviving tissues from the apical meristem and the leaf primordia (**e**), surviving tissues only in leaf primordia (**f**) and totally killed tissues (**g**), respectively. **h**, **i** and **j** are cryopreserved shoot tips (by V cryo-foil) after 14 days of post-thaw culture, showing same surviving patterns as 'e', 'f' and 'g', respectively. ST = surviving tissues; KT = killed tissues. Bars (**a**, **c**, **e**, **f** and **g**) = 100 μ m. Bars (**b** and **d**) = 50 μ m. Bars (**h**, **i** and **j**) = 1 mm.

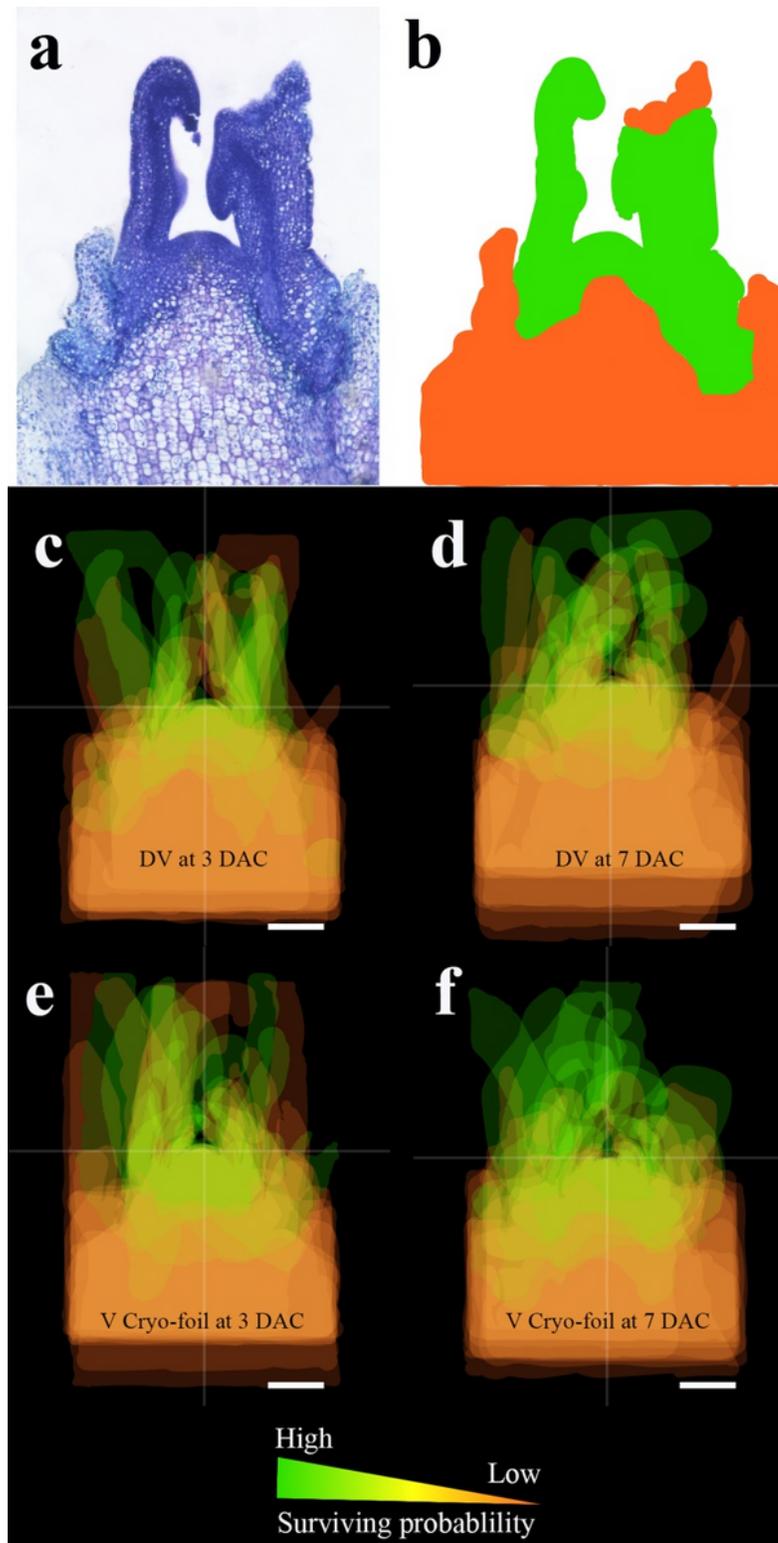


Figure 4

Histological evaluations of surviving probabilities of tissues in cryo-treated shoot tips after droplet-vitrification (Dv) and vitrification cryo-foil (V cryo-foil) cryopreservation in 'Pink Lady' apple. An apple 'Pink Lady' shoot tip cryopreserved by Dv after 3 days of post-thaw culture (3 DAC) was used to demonstrate the sample before (**a**) and after (**b**) the illustration of surviving and killed tissues with green and red colors, respectively. **c** and **d** are overlaid illustrations of 10 shoot tips cryopreserved by Dv at 3 DAC and 7 DAC, respectively. **e** and **f** are overlaid illustrations of 10 shoot tips cryopreserved by V cryo-foil at 3 DAC and 7 DAC, respectively. Bars = 100 μm .

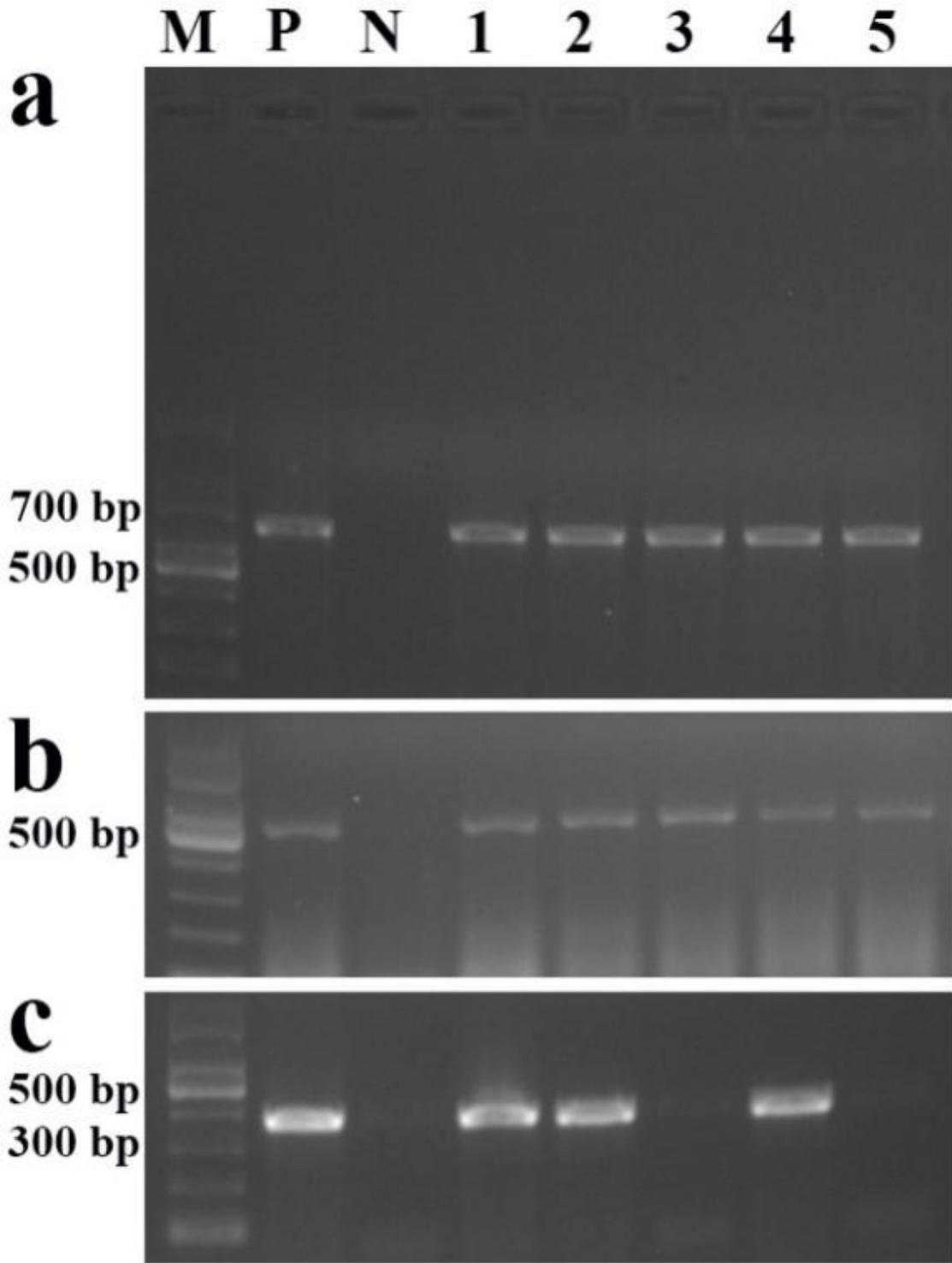


Figure 5

Detection of apple chlorotic leaf spot virus (ACLSV, **a**), apple stem grooving virus (ASGV, **b**) and apple stem pitting virus (ASPV, **c**) in 'Pink Lady' apple. Lane M = molecular marker, lane P = positive control (apple cv. 'Yanfu-8'), lane N = negative control (virus-free apple 'Yanfu-8'), lane 1 = *in vitro* apple 'Pink Lady' shoots before shoot tip cryopreservation, lanes 2-3 = shoots recovered from cryopreservation by

vitrification cryo-foil protocol after 4 months of post-thaw culture, lanes 4-5 = shoots recovered from cryopreservation by droplet-vitrification after 4 months of post-thaw culture.

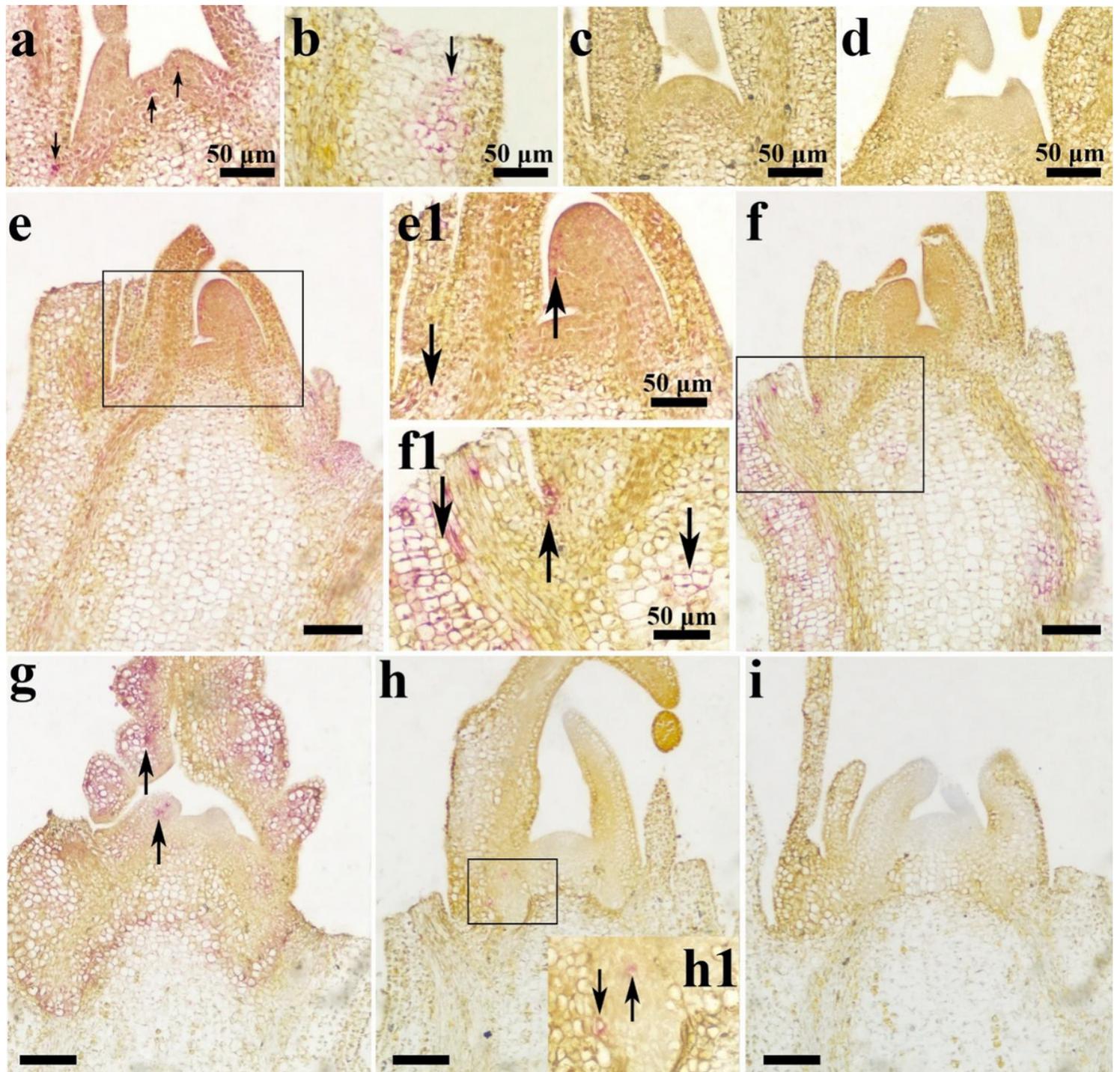


Figure 6

Immunolocalization of apple stem grooving virus (ASGV) and apple stem pitting virus (ASPV) in virus-infected shoot tips of apple 'Pink Lady'. Longitudinal sections of virus-infected 'Yanfu-8' served as the positive controls for ASGV (a) and ASPV (b). Longitudinal sections of virus-free 'Yanfu-8' served as the negative controls for ASGV (c) and ASPV (d). e and f, longitudinal sections of shoot tips of apple cv. 'Pink

Lady' stained in localization of ASGV and ASPV, respectively, before cryopreservation. **e1** and **f1** are higher magnification of area within black square in **e** and **f**, respectively. **g**, a cryopreserved 'Pink Lady' shoot tip after 7 days of post-thaw culture and stained for localization of ASGV. **h** and **i**, cryopreserved 'Pink Lady' shoot tips after 7 days of post-thaw culture and stained for localization of ASPV. **h1** is higher magnification of area within black square in **h**. Black arrows denote purple reaction of virus to immunohistology localization. Bars without numbers = 100 μ m.

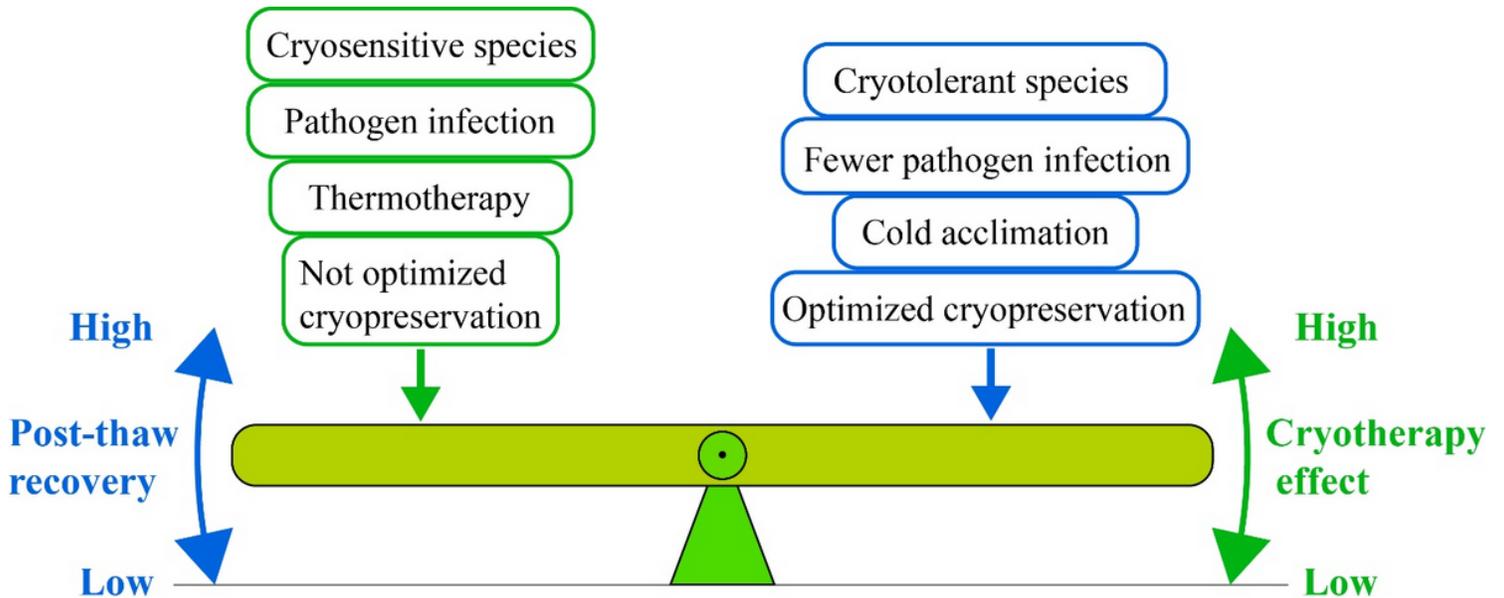


Figure 7

Seesaw effect between the post-thaw recovery and cryotherapy-effect in shoot tip cryopreservation. The study on cryosensitive species, pathogen infection, thermotherapy and not optimized cryopreservation protocols would lead to lowered post-thaw recovery after cryopreservation and improve cryotherapy-effect of pathogen eradication. On the other side, study on cryotolerant species, fewer pathogen infection, use of cold acclimation and optimized cryopreservation protocols would contribute to high post-thaw recovery, but decrease the cryotherapy effect for pathogen eradication.