

Shoot Tip Cryopreservation and Elimination of SMV and CMV Virus from Infected Stocks of *Pinellia ternata* (Thunb.) Breit.

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Additional Declarations:

Tables 1 to 3 are available in the Supplementary Files section.

Shoot Tip Cryopreservation and Elimination of SMV and CMV Virus from Infected Stocks of *Pinellia ternata* (Thunb.) Breit.

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Abstract: Cryopreservation, an effective protocol for long-term conservation, requires minimal maintenance and space. Genetic resource collection of *Pinellia ternata* (Thunb.) Breit. is essential for breeding and genetic diversity. The purpose of this study is to develop a vitrification cryopreservation protocol and virus-free technology of *P. ternata*. Shoot tips of dormant buds recovered from cryopreservation yield a regeneration rate of 75.0% and the main steps of the optimized protocol are as following: 0.8 mm-long shoot tips excised from in vitro dormant tubers subjected to cold-acclimation for 14 days, and then treated with LS for 20 min and incubated in plant vitrification solution (PVS2) for 40 min followed by plunging directly into liquid nitrogen. After rapid thawing at 38°C for 2 min, the shoot tips were unloaded in a 1.2 M sucrose solution for 20 min and then inoculated into 1/2MS medium (half-strength salts) supplement with KT 0.5 mg·L⁻¹, NAA 0.2 mg·L⁻¹, 3% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8. No obvious phenotypic variation was observed. Inter-simple sequence repeat (ISSR) detected only 0.063% variation bands in 44 cryo-derived plantlets of *P. ternata*. By means of shoot tips cryotherapy or thermotherapy in vitro plantlets at 35°C for 4 weeks followed by shoot tips culture for regenerated plants, SMV and CMV could be efficiently eradicated. Thus, cryotherapy would hold great promise as an effective approach for validly eradication of *P. ternata* viruses, and can be simultaneously applied to long-term collect *P. ternata* germplasm and produce virus-free plants.

Keyword *Pinellia ternata* (Thunb.) Breit · Cryopreservation · In vitro tuber · Virus-free · Genetic stability

Introduction

Pinellia ternata (Thunb.) Breit. is a perennial herbal plant in the Araceae family and Pillelliae rihizome, the dried tuber has been used to treat drying dampness, resolving phlegm, down-bearing counter flow to check vomiting, and dissipating masses (Bai et al. 2022; Mao and He 2020) over 2000 years in Asia countries. Pharmacological studies have also demonstrated that *P. ternata* has active medicinal effects including anti-inflammatory, anti-depressant, anti-tumor, anti-epileptic, etc. (Deng et al. 2020; Mao and He 2020). *P. ternata* is one of the top ten Chinese herbal medicines for export and the annual demand for *P. ternata* tubers in China is 5000-6000 ton, while tubers from both wild and cultivated *P. ternata* can only meet 1/3 of the market requirements. Owing to the great market demand, the natural population of *P. ternata* has been gravely harvested and badly damaged. Therefore, wild *P. ternata* has been gradually acclimatized and induced to various places in China. In field cultivation, *P. ternata* mainly propagates asexually from bulbils (Peng et al. 2007), which propagation coefficient is very low, and also lead to disease accumulation in cultivated populations, the quality and quantity of diseased plants have declined seriously (Reed et al. 1998). Thus, it is urgent to develop an effective approach for long-term storage germplasm and obtaining virus-free plants of *P. ternata*, which is critical for maintaining biodiversity, breeding and cultivation (Panis et al. 2005)

At present, vegetatively propagated plants are mostly maintained in the field or cultured in vitro under restricted conditions (Ebert and Waqainabete 2018; Reed et al. 1998). However, maintaining germplasm in field has potentially lost the valuable genetic resources due to the risk of pests and adverse weather conditions and space-limited, labor-intensive and expensive (Engelmann 2000; Engels and Ebert 2021; Roque-Borda et al. 2021). In vitro conservation needs continuously subculture, which are easily causing contamination and confusion of collections (Tanaka et al.

2004). Cryopreservation is the most reliable, cost and space-efficient long-term storage method (Benson and Reed 2008; Kaviani and Kulus 2022). Vitrification relies on exposure of explants with a highly concentrated cryoprotectant solutions to dehydrate materials, and thus all extraneous water that would otherwise freeze is removed from the cells, and both intra- and extra-cellular vitrify while avoiding the growth of intracellular ice crystals when directly immersion in LN (Benson and Reed 2008; Lambardi et al. 2000; Pawłowska and Szewczyk-Taranek 2014; Pennycooke and Towill 2000). High regeneration rates (52.9 to 90.0%) were yielded from the shoot tips of *Colocasia esculenta* (L.) Schott (Takagi et al. 1997), Mint (Hirai and Sakai 1999), *Penthorum chinense* (Zilani et al. 2022), *Musa* and *Ensete* (Panis et al. 2005), and Oil palm (Gantait et al. 2015). In the past 20 years, vitrification-based cryopreservation has been developed and using for long-term storage of various plant species (Zamecnik et al. 2021).

In recent years, viruses such as taro mosaic virus (DsMV) (Chen et al. 1996), soybean mosaic virus (SMV) (Xie et al. 2005) and cucumber mosaic virus (CMV) (Hu et al. 2004) have been identified in *P. ternata*, which extremely declined the quality and quantity of *P. ternata*. Hence, it is urgent to establish a protocol to produce virus-free plants of *P. ternata*. Currently, the most often used approaches for achieving virus-free plants are shoot tips culture, thermotherapy and thermotherapy followed by meristem culture (Kulus 2020). Recently, it has been demonstrated that plant viruses could be effectively eradicated by cryotherapy of shoot tips (Hu et al. 2004)

In this study, we have taken an initial pace to establish a vitrification cryopreservation protocol of *P. ternate*. Meanwhile, survival and regeneration rate, and virus elimination frequency of cryotherapy and traditional methods including shoot tips culture, thermotherapy and thermotherapy followed by shoot tips culture were compared.

Materials and methods

Plant materials

Tubers of *Pinellia ternata* (Thunb.) Berit. were collected in fields from Heze City of Shandong Province, China and plants regenerated in pots in a greenhouse. To induce in vitro tubers, the petioles were excised and surface sterilized with 75.0% ethanol for 10 s, then sterilized with 0.1% mercuric chloride (containing 0.1% Tween 80) for 5 min, followed by 5.0% sodium hypochlorite for 8 min, rinsed 3 times with sterile distilled water and blotted dry with sterile filter paper. Afterwards, petiole was cut off to approximately 1 cm fragment and inoculated in a flask with 1/2MS medium (half-strength salts) supplement with 3.0% (w/v) sucrose and 0.8% (w/v) agar and incubated at 25°C under dark conditions. After forming tubers, they were transferred into 12 h of light and 12 h of dark. The mature tubers refer to tubers only forming shoot tips and when it germinated and grow into seedlings for the first time named first-generation tuber and seedling. After petioles of the first-generation plantlets were cut off, tubers grew and sprout new leaves, which were named the second-generation seedlings and tubers. The shoot tips used for cryopreservation were derived from mature tubers, the first-generation (FG) tubers, and the second-generation growing (SGG) tubers and the second-generation dormant (SGD) tubers with perished leaves (Fig. 1). The diseased plants of *P.ternata* were provided by Hezheng Pharmaceutical Botanical Garden. The presence of SMV and CMV viruses was confirmed by DAS-ELISA.

Vitrification cryopreservation and regrowth of *P. ternata* shoot tips

Vitrification procedure including that shoot tips excised from *P. ternata* tuber were transferred into 1.8 mL cryovial and treated with 1 mL loading solution (2 M glycerol and 0.4 M sucrose) for 20 min at 25°C. And then, shoot tips were incubated in 1 mL PVS2 [30.0% glycerol, 15.0% ethylene glycol, 15.0% dimethyl sulfoxide (DMSO) and 0.4 M sucrose] at 25°C. The explants were rapidly immersed in LN for 2 h. And then, explants were thawed in water-bath at 38°C for 2 min, unloaded (MS+1.2 M sucrose) for 20 min at 25°C and cultured on recovery medium, which is 1/2MS supplemented with KT 0.5 mg·L⁻¹ and NAA 0.2 mg·L⁻¹.

Evaluation of major factors affecting shoot tips cryopreservation and regrowth

The major factors affecting cryopreservation and regrowth including duration of

cold acclimation for tubers, physiological stage of shoot tips, size of shoot tips, incubation period to PVS2, and whether treatment with unloading solution. For each factors the different treatments were tested as the following experiments.

Incubation time to PVS2: The first generation tuber tips of about 0.5-0.7 mm were excised as explants for cryopreservation. The incubation time to PVS2 for shoot tips was evaluated for 0, 20, 40, 90, and 120 min, respectively.

Shoot tips size: Three different sizes of shoot tips including: large (0.8~1 mm long), medium (0.5-0.7 mm long) and small (0.2-0.4 mm long) were excised from first-generation tubers and used for vitrification cryopreservation.

Physiological stage of tubers: Shoot tips approximately 0.5-0.7 mm in length were excised from three physiological states of the tuber: first generation (FG), second generation growing (SGG) and second generation dormant (SGD), respectively.

Cold-acclimated duration: Shoot tips were excised from the first-generation tubers undergoing cold-hardened at 4°C for 1 and 2 weeks, respectively. Shoot tips from non-cold-hardened treatment as a control.

Unloading: The regeneration of cryopreserved shoot tips were tested under a procedure with unloading, i.e. thawing in water bath and blotting up solution, then inoculating on recovery culture medium, and without unloading, i.e. directly inoculating cryopreserved shoot tips into recovery culture medium, respectively.

Assessments of genetic stability in cryo-derived plantlets

Phenotypes of regenerated plants after cryopreservation

Cryopreserved shoot tips were thawed, unloaded and cultured on BXJ2 medium for 8 weeks and then, cryo-derived plantlets were transferred to pots containing vermiculite and grew in a greenhouse at 16-24°C. The plantlets were irrigated and fertilized with 1/10 MS solution, and the morphological traits of individual plants were recorded, including leaf shape, leaf color, tuber color, and root color.

Genotypes of regenerated plants

Leaf samples were taken from 44 cryo-derived plantlets derived from eight shoot tips (1-8 mm). Plantlets from the same shoot tip are considered clones. DNA was

extracted by the improved CTAB method (Coelho et al. 2020). Five inter-simple sequence repeat (ISSR) markers were used for evaluating the genetic stability of the cryo-derived plantlets. The 25 μL PCR mixture consists of 12.5 μL 2xTaq PCR Master Mix (TIANCEN), 1 μL primer and 2 μL template DNA with a concentration of 25 $\text{ng}\cdot\mu\text{L}^{-1}$ in 9.5 μL double distilled water. The PCR amplification procedure was an initial denaturation at 94°C for 5 min followed by 35 cycles of 30 s at 94°C, 45 s at 49.5°C, and 2 min at 72°C, and a final extension at 72°C for 7 min. Suitable primers and annealing temperature that caused maximum reproducible bands were chosen for use (Table 1). Aliquots of 5 μL amplified PCR products was loaded into 1.5% (w/v) agarose gels for electrophoresis, followed by staining in Gel Green (Solarbio), and photographed using gel imaging system (GelDox XRTM).

Cultivation of virus-free plants

Cryotherapy

The cryopreservation protocol established above were used to eliminate the viruses.

Shoot tips culture

0.5~0.7 mm-long shoot tips were dissected from the first-generation tubers and inoculated into BX2 medium. After new tubers and plantlets regenerated, shoot tips were dissected again and cultured into plantlets.

Thermotherapy followed by shoot tips culture

The first-generation seedlings (with tubers) were placed in an illumination box and the temperature was raised gradually from 32°C to 38°C or 35°C at 1°C/d and then remained constant for 2 weeks or 4 weeks. After thermotherapy, 0.5~0.7 mm-long shoot tips were dissected and cultured into seedlings.

Statistical analysis

The experiments were conducted in a single factor experiment design and repeated at least 2 times, with 30 samples per treatment. Survival and regeneration were defined as shoot tip turned green and formed shoots, respectively. The data of ISSR-PCR amplification bands were statistically analyzed by manual band reading method. There were bands marked with "1" and no bands marked with "0" in the

electrophoresis chart. Variation bands (including deletions and additions) were considered to be inconsistent among the same lines. The data were compared by Duncan multiple-range test.

Results

Effects of major factors on survival and regeneration of cryopreservation

Shoot tips exposure duration to PVS2

Neither survival nor regeneration rate of cryo-recovery shoot tips was affected significantly when exposure to PVS2 between 20 to 60 min and the best survival and regeneration rate (both 55.7%) was obtained at 40 min (Fig. 2A). However, when incubation prolonged to 90 and 120 min, shoot tips were damaged seriously and their survival and regeneration rate decreased sharply to 23.7% (Fig. 2A).

Shoot tips size

Both highest survival and regeneration rate (61.1% and 58.3%) were most likely achieved by excising the shoot tips with 0.8-1 mm. Survival and regeneration rate were significantly decreased (both below 40.0%) when using the middle and small size of shoot tips (Fig. 2B).

Cold acclimation of in vitro plants

Cold acclimation had a different effect on survival and regeneration for shoot tips of *P. ternata* (Fig 2C). One-week cold acclimation resulted in the highest survival and regeneration rates (both 69.4%), followed by those without cold acclimation, and 2 weeks cold acclimation obviously inhibited the survival and regenerated ability of shoot tips and decreased it to the lowest level (both 26.8%) (Fig. 2C).

Physiological state of tubers

Shoot tips derived from the SGD tubers yielded a significantly high survival and regeneration rate of 75.0% after cryopreservation (Fig. 2D). No significant differences were found in survival and regeneration rate between FG tuber and the SGG tubers and the regeneration rates were 43.2% and 46.1 %, respectively (Fig. 2D).

The post-cryo shoot tips of the SGD tubers recovered in 2 weeks with tips turned green (Fig. 3A), stems and leaves started to grow in 4 weeks without callus formation

(Fig. 3B), and new roots, stems and leaves development was observed at 6 weeks of growth (Fig. 3C).

The effect of unloading

Results showed that unloading significantly effect survival and regeneration of shoot tips (Fig. 2E). The survival and regeneration rate of cryo-derived shoot tips with unloading reached approximately 60.0%, whereas without unloading shoot tips decreased sharply to 27.8% for both survival and regeneration rates. As a consequence, unloading is an absolutely necessary procedure for cryopreservation of *P. ternata* shoot tips.

Assessment of genetic stability of cryo-derived plantlets

Phenotype of regenerated plantlets

The test-tuber plantlets were transplanted into vermiculite pots, 44 regenerants grew normally, and there was no difference in leaf shape, leaf color, tuber color, and root color in the same clone. However, two different kinds of leaf shapes were observed in the different clones: one was typically heart-shaped, and the other was oval-shaped (Fig. 4). It suggested that the original tubers of *P. ternata* collected from fields were genetical admixture.

Genotypes of regenerated plantlets

Five ISSR primers generated scoreable and reproducible bands. A total of 1575 bands were amplified from 44 cryo-derived plants by these five primers (Table 2). There was a high consistency in the amplified band profiles among the plantlets within the same clones (Fig. 5), indicating the plantlets from the same clones were genetically identical. Only one of 350 bands was not identical among plantlets in clone 40-5. Overall, band variation rate is extremely only 0.063% (Table 2, Fig. 5). The difference of the band patterns from regenerated plantlets was observed between different clones (Fig. 5), which further confirmed that original tubers collected from field are genetical admixture.

Comparison of different virus-free methods

Different methods were used to eradicate the virus of *P. ternata*. The phenotypes of the in vitro seedlings were observed (Fig. 6), and the leaves were cut off and tested

by ELISA. The results showed that SMV is probably more easily eradicated compared to CMV. Cryotherapy showed a good elimination effect on CMV (57.1%), which was significantly higher than other methods (Table 3 and Fig. 7). Treatment plants at 35°C for 4 weeks was less effective in eradication of CMV (14.3%). Thermo-therapy in vitro plantlets at 38°C for 2 weeks and 35°C for 4 weeks caused the highest frequency for elimination of SMV (both 88.0%), while no significant difference was recorded between other approaches (Table 3). There was no significant difference in survival and regeneration rate either among shoot tips culture, thermo-therapy at 35°C for 2 weeks and 4 weeks or between cryotherapy and exposure plantlets at 38°C for 2 weeks. However, cryo-treated shoot tips developed after recovery culture for 10 days, which was faster than any other procedure and no callus was formed. After treatment at 35°C for 4 weeks and 35°C for 2 weeks, the in vitro seedlings were dry, and the regeneration rate decreased with the prolongation of thermo-therapy time. Consequently, combined with the analysis of the two indicators of regeneration rate and virus eradication rate, the suitable virus eradication technology system of *P. ternata* is 35°C for 4 weeks and cryotherapy. No obvious phenotypic variation was observed between plantlets regenerated from cryo-treated and those of non-treated plantlets (data not shown).

Discussion

Dormant bud was the best explant for cryopreservation of a variety of species. The first report on freezing woody plant dormant branches in liquid nitrogen using slow freezing technique was by Sakai in 1960 (Sakai 1960). Up to date, successful dormant bud cryopreservation was reported for several species, such as blackcurrant (Rantala et al. 2021), persimmon (Matsumoto et al. 2004), peony (Seo et al. 2007), aspen (Aronen and Ryyänen 2014), *Dracocephalum austriacum* (Rasl et al. 2020) and American elm (Uchendu et al. 2013). In the US Department of Agriculture-Agricultural Research Service (USDA-ARS), National Plant Germplasm System (NPGS), dormant buds were used for cryopreservation of a wide range of *Malus* (apple) accessions (Towill and Bonnard 2005), and *Salix* (Towill and Widrlechner 2004). In this study, shoot tips were dissected from growing and dormant

tubers, higher regeneration rate was obtained in dormant tubers. When the medium lost water and the plantlets perished, tuber dormancy was induced and tubers acquired strong adverse resistance. In contrast, the shoot tips from the actively growing tuber containing high water content were vulnerable to damage during cutting, freezing, and thawing process. Kwaśniewska et al. demonstrated that shoot tips from dormant buds of *Rosa pomifera* resulted in twice fold survival rate comparing to those from in vitro explants (Kwaśniewska et al. 2017). Similarly, shoot tips of persimmon dormant buds produced a survival rate of 70.0% after freezing, whereas survival rate of shoot tips from in vitro plants was as low as 30.0% (Ai 2004).

Cryopreservation causes a series of stress to explants, which may induce genetic changes in cryo-derived plantlets (Castillo et al. 2010; Martín and González-Benito 2005; Panis and Lambardi 2005). So, it is necessary to confirm whether the regenerated plants maintained genetic stability before this program is routinely applied to the long-term storage of plant germplasm. To date, there are a considerable number of studies have shown the genetic stability of cryostorage derived plants, i.e., for *chrysanthemum* (Martín et al. 2011), *Malus* (Liu et al. 2004), oil palm (Saikat et al. 2015), *Populus* hybrids (Jokipii et al. 2004) and silver birch (Ryynänen and Aronen 2005). In our study, morphological variation was not detected whereas 0.063% molecular variation alteration was found in cryo-derived plants.

Cryopreservation of shoot tips not only has already served as an effective way to protect plant germplasm but also it is recognized as a promising combined approach for virus elimination (Helliot et al. 2002; Wang et al. 2006). It is well-known that the apical meristem generally does not contain virus, as the virus in the plant mainly relies on the plant's transport tissues to spread and develop (Wang et al. 2006). There are no differentiated ducts and sieve tubes in the apical meristem, and the cells of the apical meristem are very vigorous, dividing continuously and preventing the invasion of the virus (Wang et al. 2006). When plant material is treated with LN, most differentiated cells are destroyed and die, but meristems containing less virus are able to survive. As a consequence, regenerated plants could be virus-free. The present study also showed that both SMV and CMV could be efficiently eradicated by

cryopreservation of *P. ternata* shoot tips. The first successful eradication of Plum pox virus (PPV) using cryotherapy was achieved by Brison et al. back in 1997 (Brison et al. 1997). At present, cryotherapy has been proved effective in eradicated virus of various species with a high eradication rate of 70.0%, including cucumber mosaic virus (CMV) and Banana streak virus (BSV) from bananas (Helliot et al. 2002), Potato leaf curl disease (PLRV), potato virus S (PVS) and Potato virus Y (PVY) of potato (Wang et al. 2006), apple chlorotic leaf spot virus (ACLSV) and apple stem grooving virus (ASGV) (Farhadi-Toolli et al. 2022).

When using thermotherapy in combination with shoot tips culture to eliminate the virus of plant material, the temperature and time had significant influence on virus eradication. Generally, with the longer time and higher temperature of thermotherapy treatment, the effect of virus eradication would be enhanced, however associated with the decreased regeneration rate. In the present study, regeneration rate decreased from 93.3% to 83.3% with longer duration, while eradication rate increased from 14.3% to 28.0% and 48.0%. Similarly, using the same thermotherapy, eradication rate of potato virus Y (PVY), potato virus S (PVS) and potato leaf curl virus (PLRV) was only 20.0% for 3 weeks and increased to 86.0% for 5 weeks (Gulimila-Rehemutula et al. 2022). This was further proved in lily (Yin 2022), results showed that eradication rate was 54.0% at 34°C for 1 d, 78.0% at 38°C for 1 d, and increased to 72.0% at 40°C for 1 d through thermotherapy combined with shoot tip culture to eradicate virus of lily. This validated that properly increasing the treatment temperature can increase the eradication rate of the virus.

Conclusion

This is the first report on establishing cryopreservation of *P. ternata* genetic resources. The optimum condition for cryopreservation of *P. ternata* including one-week cold acclimation at 4°C of in vitro dormant tubers, 0.8~1 mm shoot tips treated with LS, exposure to PVS2 for 40 min followed by directly immersing shoot tips into liquid nitrogen. Plants regenerated post-LN exposure have a higher level of genetic stability. Cryotherapy of *P. ternata* shoot tips can be used for virus elimination and long-term storage of germplasm. This approach will provide theory and technology support for establishment cryo-banks of germplasm of *P. ternata* and accelerating the production of healthy stock plantlets.

Highlights

1. An explant of 1cm long petioles of *P. ternata* can form an intact tuber, and the tuber can sprout and grow into plantlets.
2. The shoot tips excised from the dormant tubers in vitro are suitable for vitrification cryopreservation.
3. Highly efficient cryopreservation and Cryotherapy protocol of *P. ternata* is set up.

Author contribution statement WQD conducted experiments, analyzed data, and wrote the manuscript. JXW wrote, and revised the manuscript. SLT analyzed data. YHZ conceived and designed research and revised the manuscript. CYH, SFG and QYG conceived and designed research.

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Declarations

Conflict of interest We declare that this work was done by the authors mentioned in this article and that all responsibility for claims relating to the content of this article will be borne by the authors.

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Figures



Figure 1

The plantlets and tubers of *P. ternata* as initial materials for cryopreservation. A: The mature tubers; B: The in vitro first-generation seedlings and tubers; C: The in vitro second-generation seedlings and tubers; and D: Paraffin sections of shoot tip under four times microscope.

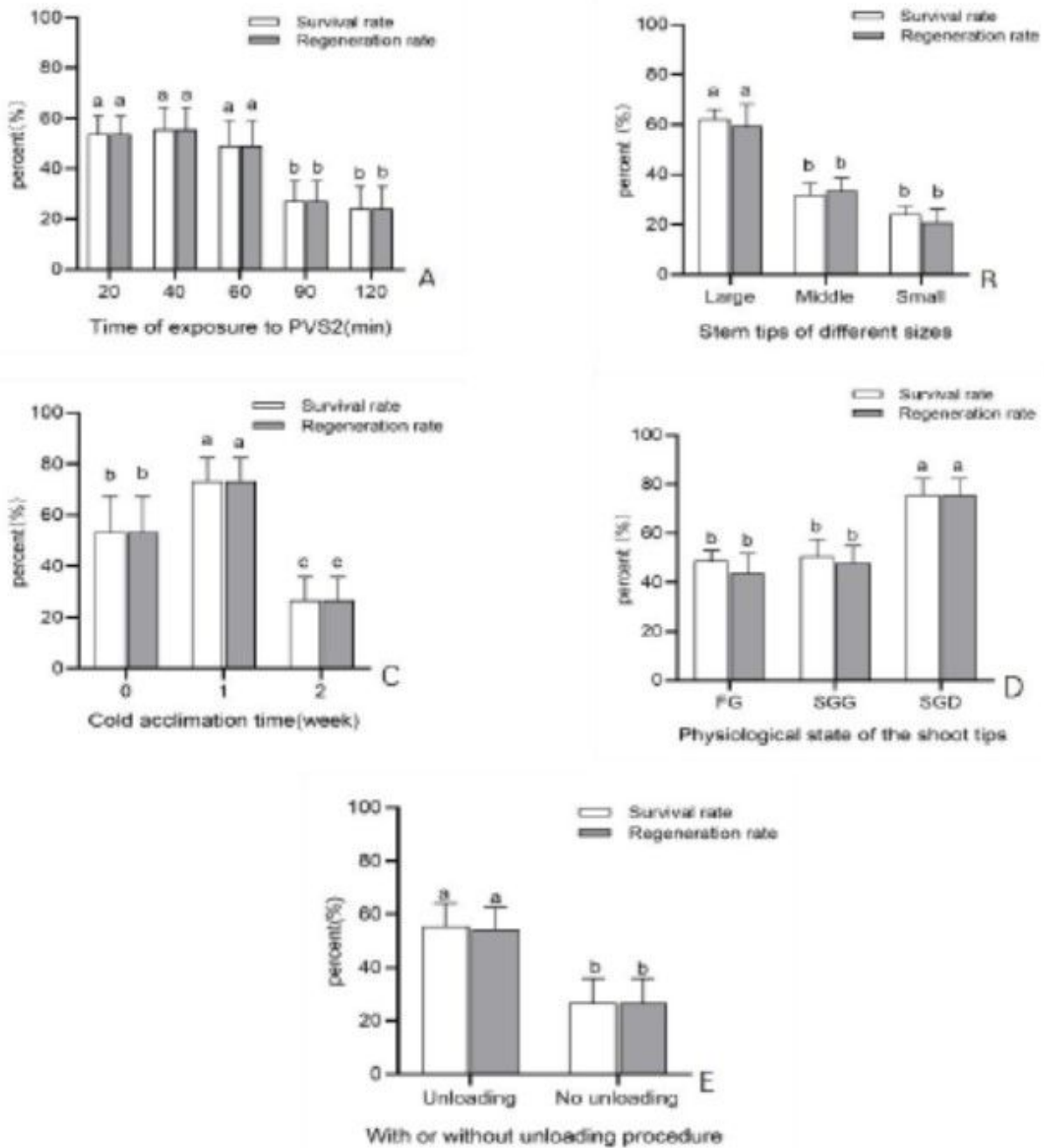


Figure 2

Effects of different treatments on the survival and regeneration rate of cryopreserved shoot tip. A: Effects of exposure length to PVS2 solution; B: Effects of shoot tip size, larger (0.8-1.0 cm), middle (0.5-0.7 cm), and small (0.2-0.4 cm); C: Effects of shoot tip from different physiological stages. FG, SGG and SGD represent the first-generation tubers, the second-generation growing tubers, and the second-generation dormant tubers, respectively; D: Effects of cold acclimation period at 4°C prior to shoot tip excision; E: Effects of procedure with and without unloading in cryopreservation.



Figure 3

Survival and regeneration of shoot tips after cryopreservation. A. The cryopreserved shoot tips turned green; B. The cryopreserved shoot tips began to grow; C. Regenerated plantlets after cryopreservation.



Figure 4

Two different leaf shapes of cryo-derived plants.

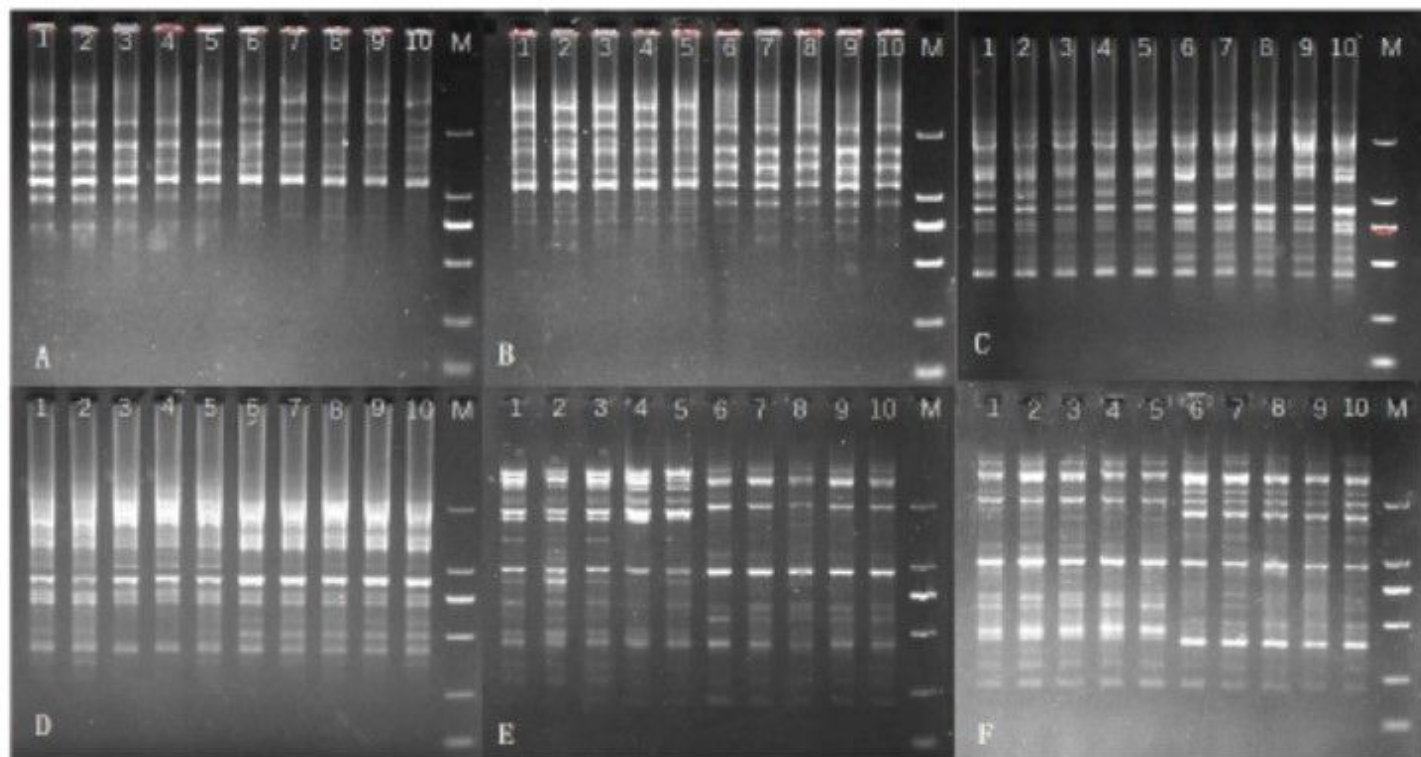


Figure 5

Profiles of ISSR marker amplification on cryo-derived plants of *P. ternata*. A and B for primer 835; C and D for primer 880; and E and F for primer 881. The lanes 1-5 in A, C and E are individual plants from clone 40-1; and the lanes 6-10 for clone 40-4. The lanes 1-5 in B, D and F are individual plants from clone 40-3; and the lines 6-10 for clone 40-6. Line M: DNA ladder.

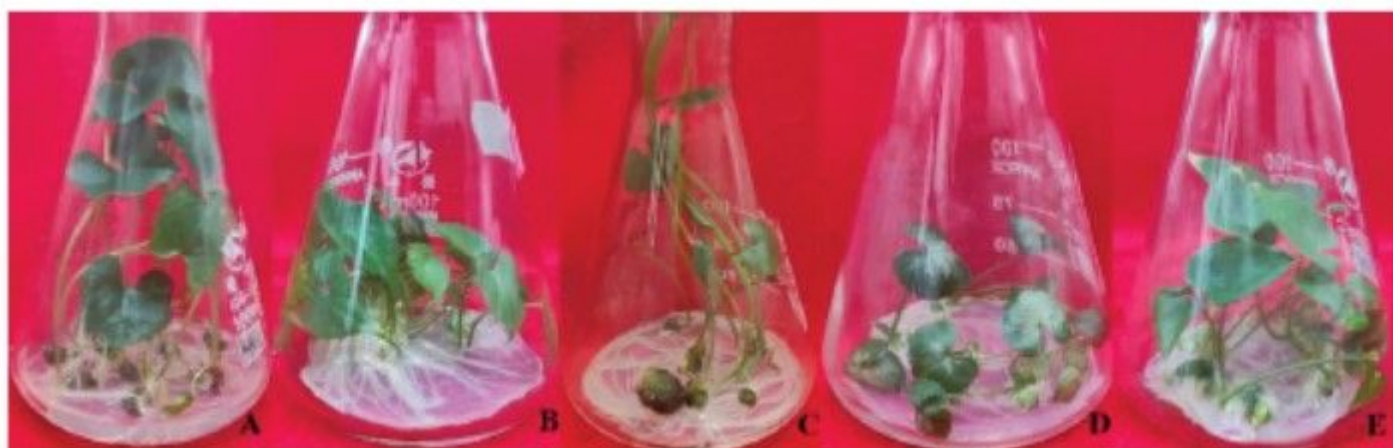


Figure 6

Regenerated plants after virus eradication by different virus eradication methods. A.Shoot tips culture; B.Cryotherapy; C.Thermotherapy in vitro plantlets at 38 °C for 2 weeks followed by shoot tips culture; D. Another thermotherapy method; E. Another thermotherapy method.

D. Thermotherapy in vitro plantlets at 35 °C for 2 weeks followed by shoot tips culture; E. Thermotherapy in vitro plantlets at 35 °C for 4 weeks followed by shoot tips culture.

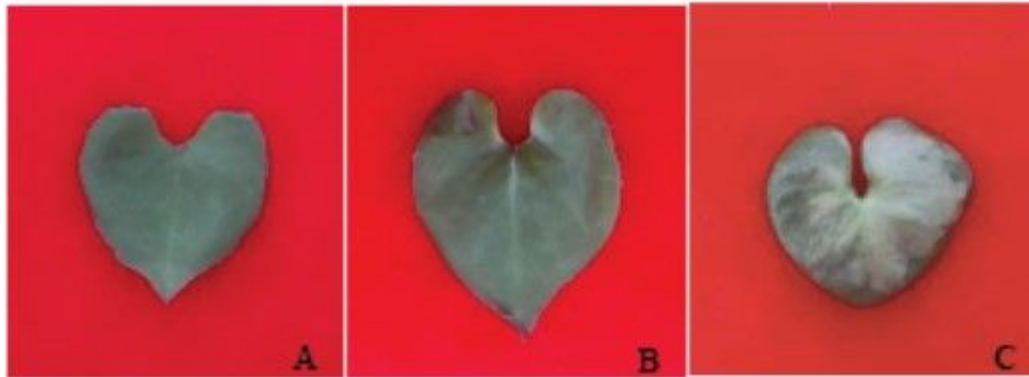


Figure 7

Phenotype of leaves after virus eradication. A. Normal leaf; B. Virus-free leaf produced by cryopreservation; C. Serious virus-infected leaf.

Supplementary Files

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