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Double-edged effects of cryogenic technique for virus eradication and preservation in shallot shoot tips

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Abstract

Plant viruses are eradicated to produce virus-free propagules for sustainable crop production, and by contrast preserved in all types of virus-related basic and applied researches. Shoot tip cryopreservation can act as a double-edged strategy, facilitating either virus eradication or preservation from regenerated plants. Herein, we tested the efficacies of shoot tip cryopreservation for virus eradication and preservation in shallot (*Allium cepa* var. *aggregatum*). *In vitro* stock shallot shoots infected with onion yellow dwarf virus (OYDV) and shallot latent virus (SLV) were thermo-treated 0, 2 and 4 weeks under constant 36 ℃ before shoot tip cryopreservation. Results showed that viruses were efficiently preserved in regenerated shoots when thermotherapy was not applied. Although thermotherapy lowered the regrowth levels of cryo-treated shoot tips, the efficiency of virus eradication increased from 5% to 54%, following the thermotherapy extended from 0 to 4 weeks. Immunolocalization of OYDV and histological observation in cryo-treated shoot tips showed the high frequency of virus preservation after the cryopreservation was due to the close invasion of virus to the apical meristem and the high proportion of survived cells. Four weeks of thermotherapy drastically decreased the distribution of OYDV as well as the percentage of survived cells in the shoot tips, thereby promoting virus eradication. Virus-free plants obtained from combining thermotherapy with cryotherapy showed significantly improved vegetative growth and bulb production. The present study well

reported how thermotherapy can act as a trigger to facilitate either the safe preservation of *Allium* viruses or the production of virus-free shallot.

Introduction

Shallot (*Allium cepa* var. *aggregatum*) is a small-bulb onion crop mainly grown in Europe, South America and Asia (Fritsch & Friesen, 2002). It is favorably consumed for its unique flavor, pungency taste and health enhancing properties (Fritsch & Friesen, 2002; Shahrajabian *et al*., 2020). Shallots are predominantly propagated by bulbs, therefore making it susceptible to the virus infection which reduces the vegetative growth, bulb yield, and eventually lead to variety degradation in *Allium* crops (Walkey & Antill, 1989; Conci *et al*., 2003; Perotto *et al*., 2010). The obtaining and use of virus-free plants through virus eradication program is currently the most effective method to combat virus problems (Faccioli & Marani, 1998; Wang *et al*. 2018a; Magyar-Tábori *et al*., 2021). As for *Allium* crops, the virus eradication was mostly focused on garlic (*Allium sativum*) by combining thermotherapy with meristem culture (Walkey *et al*., 1987; Shiboleth *et al*., 2001; Ramírez-Malagón *et al*., 2006; Pramesh & Baranwal 2015; Vieira *et al*., 2015). Shoot tip cryotherapy, as a novel method for the plant virus eradication, has also been tested in garlic (*Allium sativum*) (Kim *et al*., 2012; Vieira *et al*., 2015; Liu *et al*., 2019). It has been reported that combining the thermotherapy prior to cryotherapy proved to be the most efficient method in eliminating onion yellow dwarf virus (OYDV), leek yellow stripe virus (LYSV) and garlic common latent virus (GCLV) from garlic (Vieira *et al*., 2015). However, limited studies were made with regard to the virus elimination in shallot (Fletcher *et al*., 1998; Putri *et al*., 2019; Wang *et al*., 2021).

For virus eradication by cryotherapy, the virus-free meristem survived, leaving tissues with virus infection killed in the cryo-treatment (Brison *et al*., 1997; Wang & Valkonen 2009; Zhao *et al*., 2019). On the contrary, viruses can be preserved within the tissues of cryo-treated shoot tips when virus-infected cells survived from the cryogenic procedures (Li *et al*., 2018; Wang *et al*., 2018b). This can favor the long-term preservation of viruses over the traditional freeze-drying method in terms of reducing the risk of lowered stability and infectivity of viruses under the extended duration of preservation (Yordanova *et al*., 2000; Wang *et al*., 2018b; Zhao *et al*., 2019). Moreover, viruses recovered from the shoot tip cryopreservation can be transmitted to other hosts via mechanical inoculation or grafting (Li *et al*., 2018; Wang *et al*., 2018b), while the mechanical inoculation is the only method available in freeze-drying methods (Fukumoto & Tochinara 1998; Yordanova *et al*., 2000). To date, shoot tip cryopreservation has been tested for preservation of apple stem grooving virus (ASGV) in apple (Wang *et al*., 2018b), potato leafroll virus (PLRV), potato virus S (PVS) and potato spindle tuber viroid (PSTVd) in potato (Li *et al*., 2018), and two viroids in chrysanthemum (Li *et al*., 2019). However, the shoot tip cryogenic procedures have never been applied in shallot in terms of the eradication or the preservation of *Allium* viruses.

Since we previously optimized a droplet-vitrification protocol for cryopreservation of shallot shoot tips (Wang *et al*., 2020), the objective of the present study was therefore to assess the effects of shoot tip cryotherapy and combining thermotherapy prior to shoot tip

cryotherapy on the virus eradication and preservation in shallot. The virus distribution in the shoot tips and cell survival pattern after cryotherapy were investigated to explain the double-edged effects of the cryogenic technique in plant virus eradication / preservation. The vegetative growth and bulb production were compared between the virus-free and virus-infected shallots.

Materials and Methods

Plant materials

The Shallot genotype '10603' (*Allium cepa* var. *aggregatum*) co-infected with OYDV and SLV as confirmed in Wang *et al*. (2021) was used in the present study to evaluate the efficacies of combining thermotherapy prior to shoot tip cryopreservation on virus eradication and preservation. The *in vitro* culture was maintained on a stock shoot maintenance medium (SSMM) composed of Murashige & Skoog (1962) medium containing 30 g L^{-1} sucrose, 0.5 mg L^{-1} 6-benzylaminopurine (6-BA), 0.1 mg L^{-1} 1-naphthylacetic acid (NAA) (Fig. 1a). The medium was solidified by adding 8 g L^{-1} agar and pH of the medium was adjusted to 5.8 before autoclaving at 121 ℃ for 20 min. The culture condition was maintained at 22 ± 2 ℃ with a 16-h photoperiod provided by cool-white fluorescent tubes set at a light intensity of 50 μmol s^{-1} m^{-2}. Subculture was done every 4 weeks. Shallot 'Kverve' infected with OYDV and SLV, and the virus-free 'Kverve' were used respectively as the positive and negative control in the detection and localization of viruses.

Thermotherapy

Four-week old *in vitro* '10603' shoots were transferred to the growth chamber with same light conditions as *in vitro* cultures for thermotherapy. Temperature was set constantly at 36 ± 1 ℃. Thermotherapy was conducted for 0, 2 and 4 weeks before the cryopreservation protocol.

Cryopreservation

Shoot tips (2.0-3.0 mm in length) containing 4-5 leaf primordia (Fig. 1b) were excised from the *in vitro* '10603' shoots that had been thermo-treated for 0, 2 and 4 weeks, designated respectively as (Th0, Th2 and Th4). Excised shoot tips were subjected to the shoot tip culture (STC) as the control group, and cryopreservation protocol (Cryo). As for STC, shoot tips were cultured on SSMM under the same light conditions as the *in vitro* culture. In Cryo, a droplet-vitrification method was used as described by Wang *et al*. (2020) with minor modifications. Briefly, the excised shoot tips were incubated in SSMM overnight, followed by preculture on MS medium enriched by 0.3 M sucrose for 2 days. Precultured shoot tips were treated for 20 min at 24 ℃ with a loading solution composed of MS medium supplemented with 2.0 M glycerol and 0.6 M sucrose, and followed by exposure to plant vitrification solution 3 (PVS3) at 24 ℃ for 3 h. PVS3 contains 50% (w/v) sucrose and 50% (w/v) glycerol in MS medium (Nishizawa *et al*., 1993). After PVS3 dehydration, shoot tips

were transferred with PVS3 droplets onto aluminum foils (2 x 0.8 cm), prior to direct immersion in liquid nitrogen (LN). After immerging in LN for 1 hour, the aluminum foils were transferred into an unloading solution composed of liquid MS medium supplemented with 1.2 M sucrose at 24 °C for 20 min. Shoot tips were post-thaw cultured on MS medium containing 0.3 M sucrose for 2 days in the dark, and were then transferred to SSMM for recovery. Cryo-treated shoot tips were kept in darkness for the first 3 days, and then cultured in the same conditions as for the *in vitro* stock shoots. Subcultures was done every 4 weeks. Shoot tips that developed into normal shoots (≥ 5 mm in length) with new leaf emerged after 8 weeks of postculture were defined as regrowth (Fig. 1c).

Assessment of virus eradication / preservation efficiency

To assess virus eradication / preservation frequencies, reverse-transcription polymerase chain reaction (RT-PCR) was conducted. In the RT-PCR test, total RNA was extracted from fresh leaf tissue (0.5 g) using Spectrum™ Plant Total RNA Kit (STRN250, Sigma, USA). cDNA was further synthesized on 2 µg of total RNA using recombinant Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The PCR reaction was performed in 25 µL volume consisting of 1 µL of each primer at 10 µM, 0.5 µL of dNTPs in 10 mM, 0.2 µL of Taq DNA Polymerase with 2.5 µL of 10x buffer (10342020, Invitrogen™, ThermoFisher Scientific, USA), 2 µL of template cDNA and 17.8 µL of RNAase-free water. The primer

143 for detection of OYDV was obtained from Mahmoud *et al*. (2008) producing a specific
144 band of 601 bp, while the forward primer (5'-TGGCTACTGCTGAAGAATTGGC-3') and
145 reverse primer (5'-GCGTAACAGCGACAGACTTGG-3') designed from our previous
146 study were used to produce specific band of 238 bp for SLV detection (Wang *et al*., 2021).
147 Virus detection was firstly performed twice *in vitro* for the status of virus infection in
148 regenerated shoots. These detections were made respectively in plants after 3 and 4 months
149 of postculture. Those that tested as virus-free from were further transferred to the insect-
150 proof greenhouse to harvest bulbs after 5 months of culture. The bulbs were stored at 4 ℃
151 for 1 month and were transferred to the greenhouse again to produce new leaves. The new
152 leaves were tested by RT-PCR for the third time after two months of growth for the final
153 confirmation of their virus-free status.
154
155 **OYDV localization in shoot tips**
156 To study the virus distribution in the shoot tips before and after thermotherapy, OYDV was
157 localized in shoot tips sampled from the *in vitro* '10603' stock shoots before and after 4
158 weeks of thermotherapy. When virus-infected and -free plants regenerated respectively
159 from Th0 + Cryo and Th4 + Cryo had been cultured for 4 months, OYDV localization was
160 conducted again in their shoot tips. Virus localization was performed according to Wang
161 *et al*. (2008). Briefly, the samples were fixed in FAA (50% ethanol : formalin : acetic acid;
162 18:1:1), dehydrated with 70, 85, 95 and 100% ethanol for 2 h each, treated with xylene and

163 embedded in Paraffin. Thin paraffin sections (6 µm) were further made by rotary
164 microtome (RM 2255, Leica, Nussloch, Germany) and transferred on to slides for the
165 localization of OYDV. The well-prepared sections were first treated by removal of paraffin
166 through washing twice in xylene. Samples were then rehydrated, washed with phosphate
167 buffered saline (PBS) and blocked with PBS containing 4% bovine serum albumin.
168 Following the overnight incubation with the OYDV polyclonal antibodies (Product no.
169 160212, Bioreba, Switzerland, dilution 1: 500) to virus coat protein at 5 ℃, samples were
170 washed by rinsing with PBS for three times and incubated with anti-rabbit antibodies
171 conjugated with alkaline phosphatase (A3937 Sigma Chemical Co., USA, dilution 1:400)
172 for 30 min at room temperature. After three times of rinsing with PBS, samples were
173 stained using a freshly prepared fuchsin substrate solution (based on New Fuchsin, N0638
174 Sigma Chemical Co., USA), and purple signals were observable from the virus-infected
175 samples under the light microscope (DM6 B, Leica, Wetzlar, Germany).
176
177 **Histological observations**
178 The cell survival pattern in cryo-treated shoot tips from Th0 + Cryo and Th4 + Cryo were
179 made in histological study according to Lee *et al*. (2008). After the fixation, shoot tips were
180 stained with 1% osmium tetroxide, washed with 0.05 M phosphate buffer and dehydrated
181 in an incremental ethanol series (30%, 75%, 95% and 100%) before embedding in London
182 Resin (LR) white resin (London Resin Company, England). Semi-thin sections (1 µm)

183 were obtained with a ultramicrotome (EM UC6, Leica, Germany), mounted on glass slides
184 and stained by 0.01% toluidine blue (Sakai 1973) for histological study under the light
185 microscopy. Ultrathin sections in 60 nm were obtained with the same ultramicrotome,
186 mounted on formvar coated copper slot grids (Electron Microscopy Sciences, USA) and
187 stained with a mixture of 4% uranyl acetate (Polysciences, Inc, USA) and 1% potassium
188 permanganate for 8 min. These samples were observed for the ultrastructure of cells after
189 cryopreservation, and photographed using a transmission electron microscope (Morgagni
190 268, FEI Company B. V., The Netherlands). Samples freshly fixed without any treatment
191 were served as the positive control (survived cells), while those excised, directly immersed
192 in LN and post-cultured for 3 days were served as the negative control (killed cells). Both
193 positive and negative controls underwent the same processes as described above.
194
195 **Quantitative evaluation of OYDV signal and surviving cells**
196 For quantitative observation of the OYDV signal, assessments were made in the tissue
197 sections to measure the percentages of areas stained with purple colors before and after 4
198 weeks of thermotherapy (Th0 and Th4). Three areas designated ‘A’, ‘B’ and ‘C’ were
199 sampled in the longitudinal sections of shoot tips for comparison (Fig. 5B). For easy
200 sampling, ‘A’ represents the area in the size of 0.3 × 0.2 mm containing the apical
201 meristem and the youngest leaf primordium. ‘B’ and ‘C’ were adjacent areas in the size of
202 0.3 × 0.3 mm below A. The virus distribution level was assessed with ImageJ software

(developed by the National Institute of Health, USA, http://rsb.info.nih.gov/ij/) by measuring the percentage of areas stained with purple colors from the immunohistochemical reactions.

To evaluate the cell surviving after cryopreservation, the number of surviving cells were manually counted from the same zones ('A', 'B' and 'C') in the sections of cryo-treated shoot tips (Th0 + Cryo and Th4 + Cryo). Percentage of surviving cells from each zone was calculated.

Assessments of vegetative growth and bulb production in the virus-free plants

The virus-free plants regenerated from combining 4 weeks of thermotherapy with cryotherapy were transferred into 9-cm pots containing Jiffy-7 peat pellets (Norgro AS, Hamar, Norway) in greenhouse. Virus-infected plants derived from the *in vitro* stock cultures were used as control group. The temperature was set at 22 ± 2 ^{o}C under a 18-h photoperiod of light intensity of 200 µmol m^{-2} s^{-1}. Number of leaves, length of the longest leaf and number of the dividing plants were measured after 3 months of growth. Parameters regarding bulb production were recorded after 5 months of growth.

Experimental design and data statistical analysis

In all the STC and Cryo treatments, 10 samples were included in each treatment of three replicates. All experiments were conducted twice. Ten to twenty plants recovered from

each cryogenic treatment, depending on the number of the plants obtained, were used for virus detection by RT-PCR. Twenty-one plants were randomly selected in the comparison study of vegetative growth and bulb production in the greenhouse. As for virus localization and histological observation of survived cells, ten shoot tips were used in each treatment of two replicates. For further quantification of virus signals and surviving cells, ten shoot tips were randomly selected for the evaluations. Data of each treatment was presented as means with standard error. Least significant differences (LSD) were calculated at $P < 0.05$ by student's *t* test or one-way ANOVA with Tukey's test, depending on the number of the treatments.

Results

Effects of thermotherapy on survival of *in vitro* shoots

After 0 and 2 weeks of thermotherapy, all of the *in vitro* shallot shoots survived. When thermotherapy was extended to four weeks, about 50% of shoots showed strong symptom of hyperhydration. The hyperhydric shoots were discarded as they were not available for shoot tip excision. The leaves of shallot turned yellowish at the bottom and withered in the top after 4 weeks of thermotherapy (Fig. 1d).

Effects of Th + STC and Th + Cryo on shoot regrowth and status of virus infection

Shoot regrowth levels in Th + STC and Th + Cryo significantly decreased following the durations of thermotherapy extended from 0 to 4 weeks (Table 1). Higher regrowth levels were obtained in the STC-based treatments, from which all the regenerated plants were infected by both two viruses (Table 1). After shoot tip cryopreservation, dead shoot tips turned hyperhydric and ceased to grow, while the normal shoots could be regenerated without callus formation (Fig. 1d). In the treatment of Th0 + Cryo, 1 out of 20 tested plants was free from both viruses, while the rest were co-infected with OYDV and SLV, accounting for 95% of efficiency for virus preservation (Table 1). When thermotherapy was extended to 2 and 4 weeks prior to cryopreservation, the frequency of virus preservation decreased, as 27% (3/11) and 54% (6/11) of regenerated plants were virus-free after the treatment of Th2 + cryo and Th4 + cryo respectively (Table 1).

Virus detection

Analysis using RT-PCR for virus detection produced specific bands of 601 bp for OYDV and 238 bp for SLV (Fig. 2). When RT-PCR was applied for virus detection in the plants regenerated from Th + STC and Th + Cryo, samples showing bands of same size were considered as virus-infected, while those produced without specific bands were considered as virus-free (Fig. 2). Noticeably, single-infected plant could be obtained in regenerated plants after combining thermotherapy with cryotherapy.

Virus localization

Assisted with the immunolocalization of OYDV, purple color reactions (virus signals) were detected in the section of virus-infected positive control (Fig. 3a), whereas no such color reaction was found in the negative control (Fig. 3b). When treated without thermotherapy, purple color reactions of OYDV (Fig. 3c, c1) were easily seen from the tissues close to the apical meristem and in the lower parts of the shoot tip. Four weeks of thermotherapy led to considerably decreased level of viral signals, as OYDV signals (Fig. 3d, d1) were hardly seen in the apical dome and the lower parts of the shoot tips. In plants regenerated from Th0 + cryo followed by 4 months of subculture, OYDV was clearly revealed from the shoot tips in which viruses were still preserved (Fig. 3e), while no such signal was detected from the virus-free plants after the treatment of Th4 + cryo (Fig. 3f).

Histological observation

Cells from the freshly excised shoot tips were densely stained and presented well-preserved cytoplasm, nucleus and vacuoles (Fig. 4a). These are characteristics of living cells, presenting as the positive control in the histological study. In contrast, shoot tips were killed by a direct plunging into the LN. After three days of post culture, cells were featured with lightly-stained ruptured cytoplasm and heavily condensed nuclei, serving as the negative control (Fig. 4b). Most cells in the apical meristem survived, when shoot tips were treated by Th0 + Cryo (Fig. 4c, 4d, 4d1). Moreover, some more differentiated cells located

282 around the meristem showed preserved structure as well (Fig. 4e, e1). However, in the
283 shoot tips after the treatment of Th4 + Cryo, higher percentage of damaged cells were
284 observed (Fig. 4f). Only part of the meristem and a few layers of surrounding cells survived
285 (Fig. 4g, g1), while those located in lower part of the shoot tip were killed after Th4 + Cryo
286 (Fig.4h, h1).
287
288 **Quantitative evaluation of OYDV and surviving cells after Th0 + Cryo and Th4 +**
289 **Cryo.**
290 As was demonstrated in Fig. 5A, shoot tip cryopreservation (Th0 + Cryo) led to high level
291 of virus preservation (95%) in the regenerated shoots, while higher frequency of virus
292 eradication was produced after combining 4 weeks of thermotherapy followed by shoot tip
293 cryopreservation (Th4 + Cryo). Therefore, quantitative assessments were made regarding
294 the virus distribution before cryopreservation and the survived cells after the cryo-
295 treatment. Without thermotherapy, OYDV signals were detected from 10.5%, 16.1% and
296 14.0% of the tissues in designated zones of A, B and C respectively (Fig. 5C). The
297 measurement of survived cells after cryopreservation (Th0 + Cryo) found that 80% of the
298 cells survived in zone A in which the apical meristem was included (Fig. 5C). As for zone
299 B and C, decreased percentages of surviving cells were counted, accounting for 49% and
300 15% respectively (Fig. 5C). After 4 weeks of thermotherapy, OYDV signals were detected
301 in 1.6%, 2.2% and 2.8% of the tissues from zone A, B and C respectively (Fig. 5D).

Following the cryogenic procedures (Th4 + Cryo), 52% and 14% of the cells respectively in zone A and B survived and no surviving cells were found from zone C (Fig. 5D).

Assessments of vegetative growth and bulb production in the virus-free plants

After three months of growth in the greenhouse conditions, the number of leaves and the length of the longest leave were significantly higher in virus-free plants (Fig. 6A, Table 2). Leaves of the virus-free plants were dark green in color and did not show any chlorotic spots, while leaves of the virus-infected plants were yellowish and exhibited chlorotic spots (Fig. 6B). The number of bulbs produced per plant was 3.4 in the virus-free plants, significantly higher than the virus-infected ones (Table 2). Bulbs were produced in all of the virus-free plants sampled, while about 27% of virus-infected plants failed to produce any bulb (Fig. 6C). The bulb yield per plant was 54.8 g in the virus-free plants, significantly higher than 22.0 g produced from the virus-infected plants (Table 2). The virus-infected and virus-free plants produced similar number of dividing plants per explant (Table 2).

Discussion

Traditionally, the production of virus-free *Allium* crops relies on meristem culture or combining thermotherapy with meristem culture (Walkey *et al*., 1987; Fletcher *et al*., 1998; Pramesh & Baranwal, 2015; Vieira *et al*., 2015). In plants regenerated from the meristem culture, the frequency of virus eradication was greatly affected by the size of excised

322 explant (Faccioli & Marani, 1998; Mink *et al*., 1998; Wang *et al*., 2018b). Working on
323 garlic, Vieira *et al*. (2015) found that the virus-free frequencies were lower from the STC
324 than what were obtained from the meristem culture. Similarly in shallot, no virus-free plant
325 was produced from the 2-3 mm STC based treatments in the present study, while 10% and
326 50% of virus-free frequencies were reported respectively from the 0.5 mm meristem culture
327 and combining 4 weeks of thermotherapy with meristem culture (Wang *et al*., 2021).
328 Comparing with the traditional meristem culture, cryotherapy facilitated the use of large
329 shoot tips for high efficiencies of virus eradication (Wang & Valkonen, 2009; Zhao *et al*.,
330 2019). However, contrasting result was obtained from the present study as only 5% of
331 regenerated plants was virus-free after cryotherapy. It has been noted that virus could be
332 conserved in cryo-recovered plants, particularly for viruses that could infect the plant apical
333 meristem (Li *et al*., 2016; Tavazza *et al*., 2013; Wang *et al*., 2008; Zhao *et al*., 2019).
334 Working on garlic, Vieira *et al*. (2015) found that OYDV was detectable in the shoot tips
335 except for the first layers of cells in the meristem. Virus localization in the present study
336 also revealed close distribution of OYDV to the shallot apical meristem. These results
337 support the idea that OYDV is of high virulence in *Allium* plants (Katis *et al*., 2012; Vieira
338 *et al*., 2015) and is difficult to eradicate from shallot by cryotherapy.
339 When shoot tips are cryo-treated in LN, cells that are higher in the nucleo-cytoplasmic
340 ratio, smaller in the vacuoles size and lower in the free water content are most likely to
341 survive (Wang & Valkonen, 2009). In most plant system, meristematic cells survived from

342 cryotherapy, while more differentiated cells were killed (Helliot *et al*., 2002; Bi *et al*., 2018;
343 Li *et al*., 2016). Similar results were obtained in this study as cells of the apical meristem
344 had the highest level of survival, which was decreased the further away from the apex.
345 Unlike the surviving cells that distributed as small islands in the meristem and young leaf
346 primordia of cryo-treated banana and garlic (Helliot *et al*., 2002; Vieira *et al*., 2015), 52%
347 and 14% of cells located respectively in the more differentiated areas B and C showed
348 intact cellular structure after cryotherapy. This is consistent with the hypothesis made by
349 Kim *et al*. (2012) from which most of the upper part of the explant may survive after
350 cryotherapy and led to virus infection in regenerated garlic plants. The mechanism of
351 strong tolerance of *Allium* cells to withstand cryo-exposure is not clear. One explanation
352 may lie on the rich level of antioxidant compounds possessed in shallot and other *Allium*
353 species (Yang *et al*., 2004), since supply of exogenous antioxidants has been shown to
354 result in better regrowth for plant cryopreservation (Uchendu *et al*., 2010; Wang & Deng,
355 2004). Therefore, as was demonstrated in the Fig. 5E, the low level of virus-free frequency
356 after the cryotherapy might be the result of the close distribution of virus to the meristem
357 apex and the high level of survived cells in cryo-treated shoot tips.

358 Although cryotherapy resulted in low efficiency of virus eradication from shallot plants,
359 it favors applying this protocol as the strategy for virus preservation. In the study made by
360 Wang *et al*. (2018b) on ASGV-infected apple 'Gala', since ASGV was recalcitrant to
361 eradicate by cryotherapy, high efficiency and reliability of ASGV preservation was

362 achieved using shoot tip cryopreservation. Similar results were also reported by Li *et al*.
363 (2018) for the long-term preservation of PVS and PSTVd in Potato. In spite of the lowered
364 virus concentration in cryo-derived shoots due to the reduced number of survived cells, it
365 could be recovered following the subsequent subculture (Wang *et al*., 2018; Li *et al*., 2018).
366 In the present study, similar distribution of OYDV was revealed in the cryo-treated shoot
367 tips that had been sub-cultured for 4 months. Although the evaluation for cryopreserved
368 virus was not made as Li *et al*. (2018) and Wang *et al*. (2018), the results obtained from
369 the present study support the use of shoot tip cryopreservation for the long-term
370 preservation of OYDV and SLV in shallot.

371 Thermotherapy proves effective in improving the efficiency of virus eradication (Wang
372 *et al*. 2018a). The extent of virus eradication is positively affected by the duration of
373 thermotherapy (Tan *et al*., 2010; Wang *et al*., 2018a; Zhao *et al*., 2018). In this study,
374 thermotherapy of shallot shoots for 4 weeks followed by cryotherapy produced higher
375 frequency of plants free from viruses than those obtained after the 0- and 2-week
376 thermotherapy. It has been substantiated that thermotherapy inhibits virus replication and
377 its movement towards the meristematic cells (Cooper & Walkey, 1978; Mink *et al*., 1998).
378 It also triggers the virus-induced RNA silencing, which consequently leads to the reduced
379 level of virus concentration (Liu *et al*., 2015, 2016; Wang *et al*., 2008). Clear reduction of
380 virus signals in the meristem was found in the thermo-treated *Rubus* infected with raspberry
381 bushy dwarf virus (RBDV) (Wang *et al*., 2008) and the apple 'Gala' infected with ASGV

(Zhao *et al*., 2018). Consistent with those reported by Wang *et al*. (2008) and Zhao *et al*. (2018), significant reduction of OYDV signal was detected from shallot shoot tips after 4 weeks of thermotherapy.

Thermotherapy induced stress to plants and greatly affected the survival of *in vitro* shoots as well as the regrowth of shoot tips when combined with cryotherapy (Wahid *et al*., 2007; Wang *et al*., 2008; Zhao *et al*., 2018). The enlarged sizes of cells and vacuoles are main features of subcellular alterations in cells under thermotherapy (Wang *et al*., 2008; Wang *et al*., 2021). Those changes were quantified in thermo-treated raspberry, in which two-fold and six-fold increases were found respectively for the cell size and the vacuole volume in the youngest leaf primordium (Wang *et al*., 2008). With enlarged vacuoles after thermotherapy, the increased free water therein may lead to more crystallization from freezing (Hills & Nott, 1999; Wang *et al*., 2008), thus reducing the tolerance of cells to cryotherapy. The results obtained from the present study clearly showed that four weeks of thermotherapy significantly led to decreased level of cell survivals in cryo-treated shoot tips, in which small islands of surviving cells were formed as those observed from the cryo-treated *Musa* and garlic (Helliot *et al*., 2002; Vieira *et al*., 2015). As was shown in the Fig. 5E, the reduced cell survival was in synergism with decreased virus distribution, thus contributing to the improved virus-free frequency after combining thermotherapy with cryotherapy.

In the current study, virus-free plants obtained from combining thermotherapy with cryotherapy were evaluated under greenhouse conditions. In onion, OYDV causes great loss in bulb yield and seed production (Bos 1982; Katis *et al*., 2012). SLV, while being mostly symptomless in shallot and other *Allium* crops, can act synergistically with potyviruses like OYDV, leading to more severe yield losses (Katis *et al*., 2012). Therefore, the eradication of those viruses will free plants from the adverse influences of virus infection, leading to the improved agronomic traits. In the present study, the level of vegetative growth and the bulb production were significantly higher in virus-free plants as compared with plants infected by OYDV and SLV. Similar results were also reported by Walkey & Antill (1989) and Ramírez-Malagón et al. (2006) in which the virus-free garlics had significantly longer leaves and bigger cloves. These improved performances of virus-free plants emphasized the necessity of using virus-eradication programs as suggested by Conci *et al*. (2015) to combat the problem of virus disease in *Allium* crops.

In conclusion, OYDV and SLV were efficiently preserved in shoot tips after cryotherapy, supporting the use of shoot tip cryopreservation for the long-term preservation of these viruses. Combining four weeks of thermotherapy prior to cryotherapy proved applicable for virus eradication as 54% of regenerated plants were virus-free, similar to the 50% obtained from combining thermotherapy with meristem culture (Wang *et al*., 2021). Nevertheless, applying thermotherapy prior to cryotherapy can be considered together with the conventional meristem-based method, depending on the availability and capability of

an eradication program, to produce virus-free shallot. Virus-freed shallot was superior in terms of the vegetative growth and bulb production. Thus, thermotherapy can act as a trigger in shallot cryogenic techniques for different purpose, i.e., the long-term preservation of *Allium* viruses for fundamental and applied research, or the virus eradication for the commercial production.

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Data Availability Statement

441 The data that support the findings of this study are available from the corresponding author
442 upon reasonable request.
443
444 **References**
445 Bi WL, Hao XY, Cui ZH, Pathirana R, Volk GM, Wang QC, 2018. Shoot tip cryotherapy
446 for efficient eradication of grapevine leafroll-associated virus-3 from diseased
447 grapevine *in vitro* plants. *Annals of Applied Biology* 173, 261-70.
448 Bos L, 1982. Viruses and virus diseases of *Allium* species. *Acta Horticulturae* 127, 11-30.
449 Brison M, de Boucaud MT, Pierronnet A, Dosba F, 1997. Effect of cryopreservation on
450 the sanitary state of a cv *Prunus* rootstock experimentally contaminated with Plum Pox
451 Potyvirus. Plant Sci 123, 189-96.
452 Conci VC, Canavelli A, Lunello P *et al*., 2003. Yield losses associated with virus-infected
453 garlic plants during five successive years. *Plant Disease* 87, 1411-5.
454 Conci V, Perotto M, Cafrune E, Lunello P, 2005. Program for intensive production of virus-
455 free garlic plants. *Acta Horticulturae* 688, 195-200.
456 Cooper VC, Walkey D, 1978. Thermal inactivation of cherry leaf roll virus in tissue
457 cultures of *Nicotiana rustica* raised from seeds and meristem-tips. *Annals of Applied*
458 *Biology* 61, 361-72.

459 Faccioli G, Marani F, 1998. Virus elimination by meristem tip culture and tip micrografting.
460 In: Hadidi A, Khetarpal RH, Koganezawa H, eds. *Plant Virus Disease Control.* St Paul,
461 MN, 346-80.
462 Fletcher P, Fletcher J, Lewthwaite S, 1998. *In vitro* elimination of onion yellow dwarf and
463 shallot latent viruses in shallots (*Allium cepa* var. *ascalonicum* L.). *New Zealand*
464 *Journal of Crop and Horticultural Science* 26, 23-6.
465 Fritsch R, Friesen N, 2002. Evolution, domestication and taxonomy. In: Rabinowitch HD,
466 Currah L, eds. *Allium Crop Science: Recent Advances*. CABI publishing, Oxon, UK, 5-
467 30.
468 Fukumoto F, Tochinara H, 1998. Preservation of purified peanut stripe and turnip mosaic
469 potyviruses by freezing and freeze-drying. *Journal of Phytopathology* 146, 89-95.
470 Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Frison E, 2002. Cryopreservation
471 for the elimination of cucumber mosaic and banana streak viruses from banana (*Musa*
472 spp.). *Plant Cell Reports* 20, 1117-22.
473 Hills BP, Nott KP, 1999. NMR studies of water compartmentation in carrot parenchyma
474 tissue during drying and freezing. *Applied Magnetic Resonance* 17, 521–35.
475 Katis NI, Maliogka VI, Dovas CI, 2012. Viruses of the genus *Allium* in the Mediterranean
476 region. In: Loebenstein G, Lecoq H, eds. *Advances in VIRUS RESEARCH, Viruses and*
477 *Virus diseases of Vegetables in the Mediterranean Basin*. Academic Press, USA, 163-
478 208.

479 Kim HH, Popova E, Shin DJ *et al*., 2012. Cryobanking of Korean *Allium* germplasm
480 collections: results from a 10 year experience. *CryoLetters* 33, 45-57.
481 Lee Y, Derbyshire P, Knox JP, Hvoslef-Eide AK, 2008. Sequential cell wall
482 transformations in response to the induction of a pedicel abscission event in *Euphorbia*
483 *pulcherrima* (poinsettia). *the Plant Journal* 54, 993-1003.
484 Li BQ, Feng CH, Hu LY, Wang MR, Wang QC, 2016. Shoot tip culture and
485 cryopreservation for eradication of Apple stem pitting virus (ASPV) and Apple stem
486 grooving virus (ASGV) from apple rootstocks 'M9' and 'M26'. *Annals of Applied*
487 *Biology* 168, 142-50.
488 Li JW, Hosokawa M, Nabeshima T, Motoki K, Yamada H, Wang QC, 2019.
489 Cryopreservation of viroid-infected chrysanthemum shoot tips. *Scientia Horticulturae*
490 244, 1-9.
491 Li JW, Wang MR, Chen HY *et al*., 2018. Long-term preservation of potato leafroll virus,
492 potato virus S and potato spindle tuber viroid in cryopreserved shoot tips. *Applied*
493 *Microbiology and Biotechnology* 102, 10743-54.
494 Liu J, Zhang X, Zhang F *et al*., 2015. Identification and characterization of microRNAs
495 from *in vitro*-grown pear shoots infected with Apple stem grooving virus in response to
496 high temperature using small RNA sequencing. *BMC Genomics* 16, 945.

497 Liu J, Zhang X, Yang Y *et al*., 2016. Characterization of virus-derived small interfering
498 RNAs in Apple stem grooving virus-infected *in vitro*-cultured *Pyrus pyrifolia* shoot tips
499 in response to high temperature treatment. *Virology Journal* 13, 166.
500 Liu XX, Mou SW, Cheng ZH, 2019. Effect of Cryopreservation on Plant Growth, Bulb
501 Characteristics, and Virus Reduction of Garlic (*Allium Sativum* L.). *Cryoletters* 40, 322-
502 32.
503 Magyar-Tábori K, Mendler-Drienyovszki N, Hanász A, Zsombik L, Dobránszki J, 2021.
504 Phytotoxicity and Other Adverse Effects on the *In Vitro* Shoot Cultures Caused by Virus
505 Elimination Treatments: Reasons and Solutions. *Plants* 10, 670.
506 https://doi.org/10.3390/plants10040670
507 Mahmoud SY, Abo-El Maaty SA, El-Borollosy AM, Abdel-Ghaffar MH, 2008.
508 Identification of Onion yellow dwarf potyvirus as one of the major viruses infecting
509 garlic in Egypt. *International Journal of Virology* 4, 1-13.
510 Mink G, Wample R, Howell W, 1998. Heat treatment of perennial plants to eliminate
511 phytoplasmas, viruses and viroids while maintaining plant survival. In: Hadidi A,
512 Khetarpal RH, Koganezawa H, eds. *Plant Virus Disease Control*. St Paul, MN, 332-45.
513 Murashige T, Skoog F, 1962. A revised medium for rapid growth and bio assays with
514 tobacco tissue cultures. *Physiologia Plantarum* 15, 473-97.

515 Nishizawa S, Sakai A, Amano Y, Matsuzawa T, 1993. Cryopreservation of asparagus
516 (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant
517 regeneration by vitrification. *Plant Science* 91, 67-73.
518 Perotto MC, Cafrune EE, Conci VC, 2010. The effect of additional viral infections on
519 garlic plants initially infected with *Allexiviruses*. *European Journal of Plant Pathology*
520 126, 489-95.
521 Pramesh D, Baranwal V, 2015. Production of virus-free garlic (*Allium sativum* L.) through
522 meristem tip culture after solar or hot air treatment of cloves. *The Journal of*
523 *Horticultural Science and Biotechnology* 90, 180-6.
524 Putri PH, Hidayat SH, Dinarti D, 2019. Elimination of Potyvirus and Carlavirus from
525 Infected shallot bulbs. *Journal of Applied Horticulture* 21, 42-6.
526 Ramírez-Malagón R, Pérez-Moreno L, Borodanenko A, Salinas-González G, Ochoa-Alejo
527 N, 2006. Differential organ infection studies, potyvirus elimination, and field
528 performance of virus-free garlic plants produced by tissue culture. *Plant Cell Tissue and*
529 *Organ Culture* 86, 103-10.
530 Sakai WS, 1973. Simple method for differential staining of paraffin embedded plant
531 material using toluidine blue O. *Stain Technology* 48, 247-9.
532 Shahrajabian MH, Sun W, Cheng, Q, 2020. Chinese onion, and shallot, originated in Asia,
533 medicinal plants for healthy daily recipes. *Notulae Scientia Biologicae* 12, 197-207.

534 Shiboleth YM, Galon A, Koch M, Rabinowitch HD, Salomon R, 2001. Molecular
535 characterisation of Onion yellow dwarf virus (OYDV) infecting garlic (*Allium sativum*
536 L.) in Israel: thermotherapy inhibits virus elimination by meristem tip culture. *Annals*
537 *of Applied Biology* 138, 187-95.
538 Tan RR, Wang LP, Hong N, Wang GP, 2010. Enhanced efficiency of virus eradication
539 following thermotherapy of shoot-tip cultures of pear. *Plant Cell Tissue and Organ*
540 *Culture* 101, 229-35.
541 Tavazza R, Lucioli A, Benelli C, Giorgi D, D'Aloisio E, Papacchioli V, 2013.
542 Cryopreservation in artichoke: towards a phytosanitary qualified germplasm collection.
543 *Annals of Applied Biology* 163, 231-241.
544 Uchendu EE, Muminova M, Gupta S, Reed BM, 2010. Antioxidant and anti-stress
545 compounds improve regrowth of cryopreserved *Rubus* shoot tips. *In Vitro Cellular &*
546 *Development Biology – Plant* 46, 386-93.
547 Vieira RL, Silva ALD, Zaffari GR, Steinmacher DA, Fraga HPDF, Guerra MP, 2015.
548 Efficient elimination of virus complex from garlic (*Allium sativum* L.) by cryotherapy
549 of shoot tips. *Acta Physiologiae Plantarum* 37, 1-11.
550 Wahid A, Gelani S, Ashraf M, Foolad MR, 2007. Heat tolerance in plants: an overview.
551 *Enviromental and Experimental Botany* 61, 199-223.
552 Walkey D, Antill D, 1989. Agronomic evaluation of virus-free and virus-infected garlic
553 (*Allium sativum* L.). *Journal of Horticultural Science* 64, 53-60.

554 Walkey D, Webb M, Bolland C, Miller A, 1987. Production of virus-free garlic (*Allium*
555 *sativum* L.) and shallot (*A. ascalonicum* L.) by meristem-tip culture. *Journal of*
556 *Horticultural Science* 62, 211-20.
557 Wang MR, Cui ZH, Li JW, Hao XY, Zhao L, Wang QC, 2018a. In vitro thermotherapy-
558 based methods for plant virus eradication. *Plant Methods* 14, 87.
559 Wang MR, Hamborg Z, Blystad DR, Wang QC, 2021. Combining thermotherapy with
560 meristem culture for improved eradication of onion yellow dwarf virus and shallot latent
561 virus from infected *in vitro* - cultured shallot shoots. *Annals of Applied Biology* 178,
562 442-9.
563 Wang MR, Yang W, Zhao L *et al*., 2018b. Cryopreservation of virus: a novel
564 biotechnology for long-term preservation of virus in shoot tips. *Plant Methods* 14, 47.
565 Wang MR, Zhang Z, Zámečník J *et al*., 2020. Droplet-vitrification for shoot tip
566 cryopreservation of shallot (*Allium cepa* var. *aggregatum*): effects of PVS3 and PVS2
567 on shoot regrowth. *Plant Cell Tissue and Organ Culture* 140, 185-95.
568 Wang Q, Cuellar WJ, Rajamäki ML, Hirata Y, Valkonen JPT, 2008. Combined
569 thermotherapy and cryotherapy for efficient virus eradication: relation of virus
570 distribution, subcellular changes, cell survival and viral RNA degradation in shoot tips.
571 *Molecular Plant Pathology* 9, 237-50.
572 Wang QC, Valkonen JPT, 2009. Cryotherapy of shoot tips: novel pathogen eradication
573 method. *Trends in Plant Science* 14, 119-22.

574 Wang ZC, Deng XX, 2004. Cryopreservation of shoot-tips of citrus using vitrification:
575 effect of reduced form of glutathione. *CryoLetters* 25, 43-50.
576 Wu H, Qu X, Dong Z *et al*., 2020. WUSCHEL triggers innate antiviral immunity in plant
577 stem cells. *Science* 370, 227-31.
578 Yang J, Meyers KJ, van der Heide J, Liu RH, 2004. Varietal differences in phenolic content
579 and antioxidant and antiproliferative activities of onions. *Journal of Agricultural and*
580 *Food Chemistry* 52, 6787-93.
581 Yordanova A, Stoimenova E, Donev T, 2000. Prediction of preservation of freeze-dried
582 cucumber mosaic virus. *Biotechnology Letters* 22, 1779-82.
583 Zhao L, Wang MR, Cui ZH, Chen L, Volk GM, Wang QC, 2018. Combining
584 Thermotherapy with Cryotherapy for Efficient Eradication of Apple stem grooving
585 virus from Infected In-vitro-cultured Apple Shoots. *Plant Disease* 102, 1574-80.
586 Zhao L, Wang MR, Li JW, Cui ZH, Volk GM, Wang QC, 2019. Cryobiotechnology: a
587 double-edged sword for obligate plant pathogens. *Plant Disease* 103, 1058-67.
588

589 Figure legends

590 **Figure 1** Explants and shoot regeneration in shallot shoot tip cryopreservation. (a)
591 Virus-infected *in vitro* cultures of shallot '10603'. (b) Shoot tip excised for
592 cryopreservation. (c) Virus-infected *in vitro* shoots before thermotherapy (in the left)

593 and after 4 weeks of thermotherapy (in the right). (d) Shoot regenerated after
594 cryopreservation.

595 **Figure 2** Detection of onion yellow dwarf virus (OYDV) (a) and shallot latent virus
596 (SLV) (b) in shallot '10603' by reverse-transcription polymerase chain reaction
597 (RT-PCR). Lane M=molecular marker; lane P = positive control (OYDV and
598 SLV-infected shallot 'Kverve'); lane N = negative control (virus-free shallot
599 'Kverve'); lane 1 = OYDV and SLV co-infected '10603' stock shoots analyzed
600 by RT-PCR; lanes 2 and 3 = Detection of viruses in plantlets regenerated from
601 cryotherapy (Th0 + Cryo); lanes 4-10 = Detection of viruses in plantlets
602 regenerated from combining 4 weeks of thermotherapy with cryotherapy (Th0 +
603 Cryo).

604 **Figure 3** Immunolocalization of onion yellow dwarf virus (OYDV) in the virus-
605 infected and virus-free shallot shoot tips of '10603'. Virus-infected cells show
606 purple color reaction in response to staining by fuchsin substrate solution, while
607 healthy cells do not. (a) Longitudinal section of OYDV-infected shallot 'Kverve'
608 served as the positive control and (b) OYDV-free shallot 'Kverve' served as the
609 negative control. (c) Longitudinal section of shallot '10603' shoot tip excised before
610 thermotherapy. (c1) closer views of black squares in 'c'. (d) Longitudinal section of
611 shallot '10603' shoot tip prepared after 4 weeks of thermotherapy. (d1) closer
612 views of black squares in 'd'. (e) Longitudinal section of virus-infected shallot

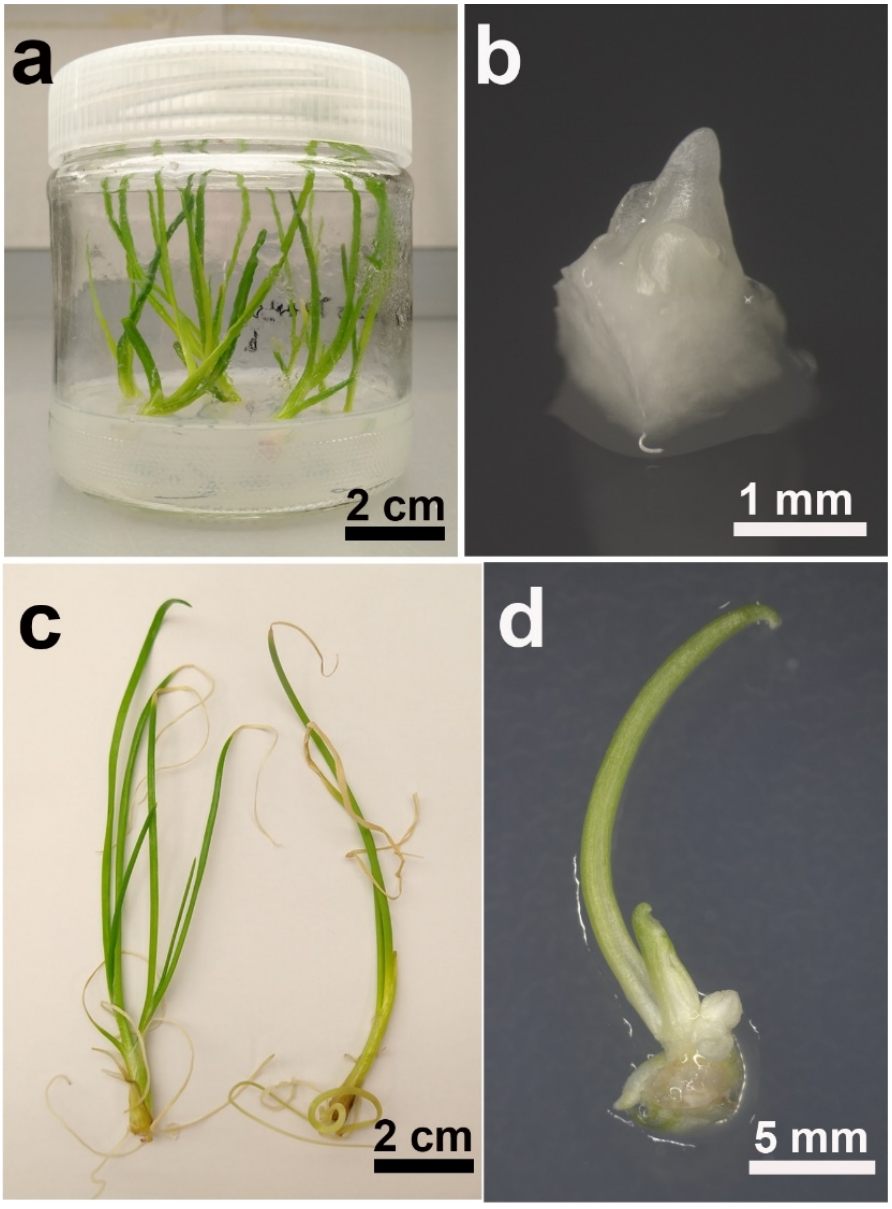
613 '10603' shoot tip regenerated from the treatment Th4 + Cryo followed by 4 months
614 of subculture. (f) Longitudinal section of virus-free shallot '10603' shoot tip
615 regenerated from the treatment Th4 + Cryo followed by 4 months of subculture.
616 Black arrows denote purple reaction of virus from the immunohistology staining.
617 Bars without numbers = 500 μm.

618 **Figure 4** Histological observations on cell survival in cryopreserved shoot tips of
619 shallot '10603'. (a) Cells from freshly excised shoot tips serve as positive control.
620 (b) Cells from shoot tip that was directly plunged into liquid nitrogen serve as
621 negative control. (c) A shallot shoot tip exposed to cryotherapy and cultured for 3
622 days. (d) Closer view of meristem cells of cryo-treated shoot tip in c**.** (d1)**,**
623 Ultrastructural observation of cell in d**.** (e) Closer view of cells in the black square
624 of cryo-treated shoot tip in c. (e1) Ultrastructural observation of cell in e. (f) A shallot
625 shoot tip treated by combining 4 weeks of thermotherapy with cryotherapy and
626 cultured for 3 days. (g) Closer view of meristem cells of cryo-treated shoot tip in f**.**
627 (g1) Ultrastructural observation of cell in g**.** (h) Closer view of cells in the black
628 square of cryo-treated shoot tip in f. (h1) Ultrastructural observation of cell in h.
629 Black and white arrows denote surviving and damaged cells, respectively.

630 **Figure 5** Combining thermotherapy with cryotherapy for preservation and
631 eradication of viruses. (A) Percentage of various virus infection status in plants
632 after combining thermotherapy with cryopreservation. (B) Three designated

areas ‘A’, ‘B’ and ‘C’ in the sections of shoot tip used for measurement of OYDV signals and survived cells. (C) Percentages of areas stained from the localization of OYDV in the shoot tips before thermotherapy (Th0) and percentages of surviving cells after the following cryotherapy (Th0+Cryo) in three designated zones. (D) Percentages of areas stained from the localization of OYDV in the shoot tips after 4 weeks of thermotherapy (Th4) and percentages of surviving cells after the following cryotherapy (Th4+Cryo) in three designated zones. (E) Schematic illustration of cryopreservation (Th0+Cryo) and combining 4 weeks of thermotherapy with cryopreservation (Th4+Cryo) for virus elimination. Data represent the means ± SE of ten samples.

Figure 6 Comparison of vegetative growth and bulb production between *in vitro* virus-infected (virus +) shallots and virus-free (virus -) shallots obtained from combining thermotherapy with cryotherapy. (A) Comparison of vegetative growth and (B) leaf color of virus-infected shoots with virus-free shoots from combining thermotherapy with cryotherapy after 3-month culture in the greenhouse. (C) Comparison of bulbs produced between the virus-free and the virus-infected plants. Bulbs from the lines a-e were from the virus-free plants while bulbs in the lines f-j were the virus-infected ones. Virus-infected plants g1, h2, i2 and i3 produced no bulb in the assessment.



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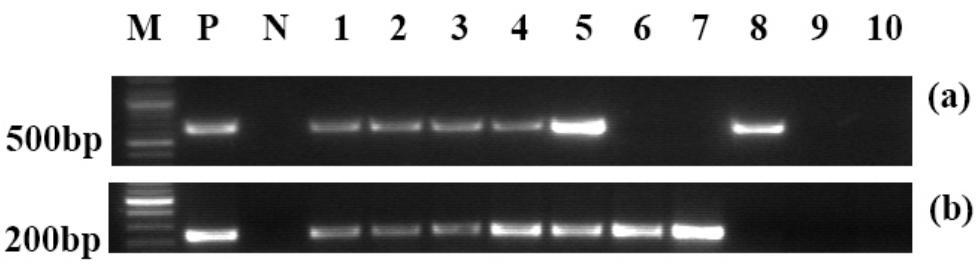

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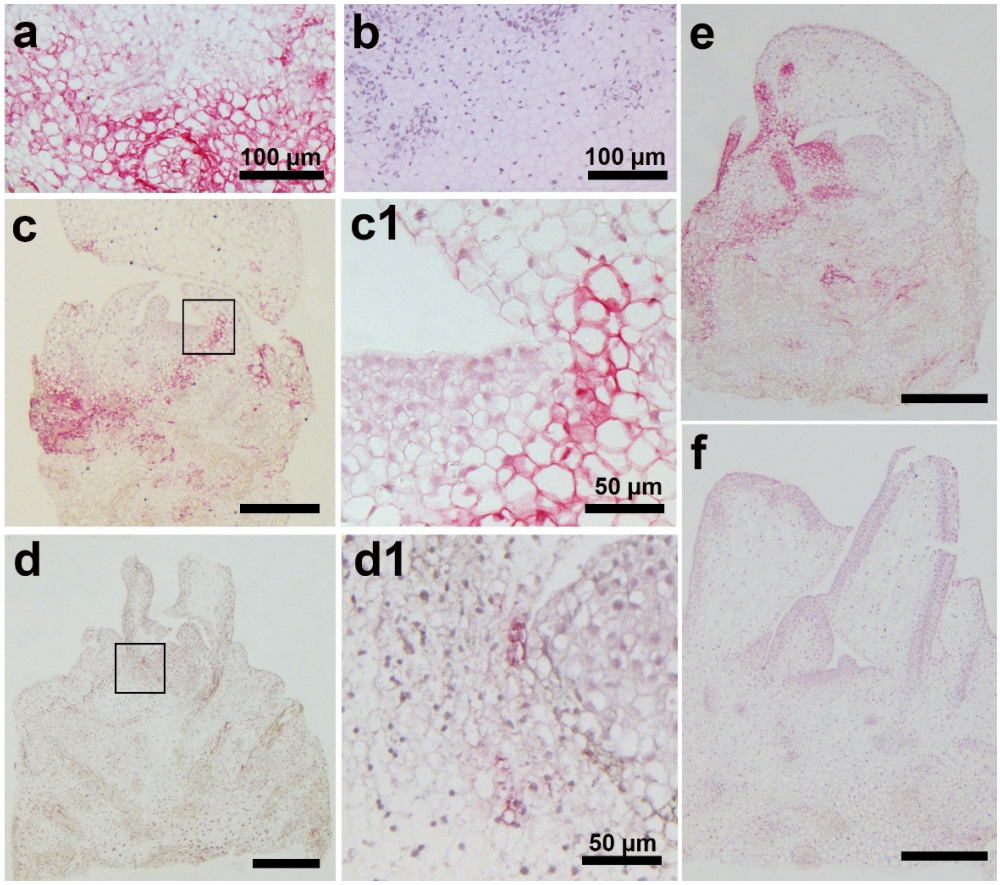

Figure 3 Immunolocalization of onion yellow dwarf virus (OYDV) in the virus-infected and virus-free shallot shoot tips of '10603'. Virus-infected cells show purple color reaction in response to staining by fuchsin substrate solution, while healthy cells do not. (a) Longitudinal section of OYDV-infected shallot 'Kverve' served as the positive control and (b) OYDV-free shallot 'Kverve' served as the negative control. (c) Longitudinal section of shallot '10603' shoot tip excised before thermotherapy. (c1) closer views of black squares in 'c'. (d) Longitudinal section of shallot '10603' shoot tip prepared after 4 weeks of thermotherapy. (d1) closer views of black squares in 'd'. (e) Longitudinal section of virus-infected shallot '10603' shoot tip regenerated from the treatment Th4 + Cryo followed by 4 months of subculture. (f) Longitudinal section of virus-free shallot '10603' shoot tip regenerated from the treatment Th4 + Cryo followed by 4 months of subculture. Black arrows denote purple reaction of virus from the immunohistology staining. Bars without numbers = 500 µm.

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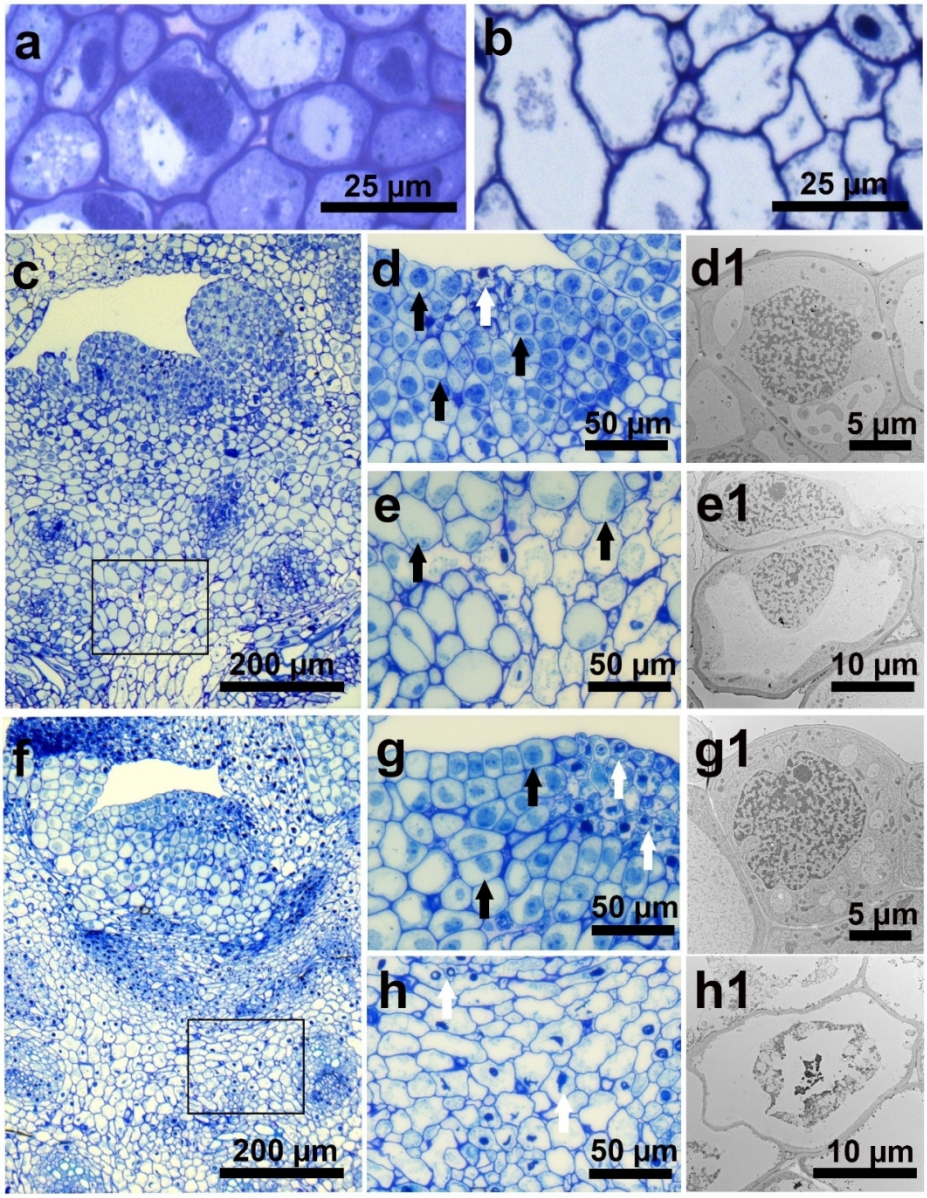

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146x188mm (220 x 220 DPI)

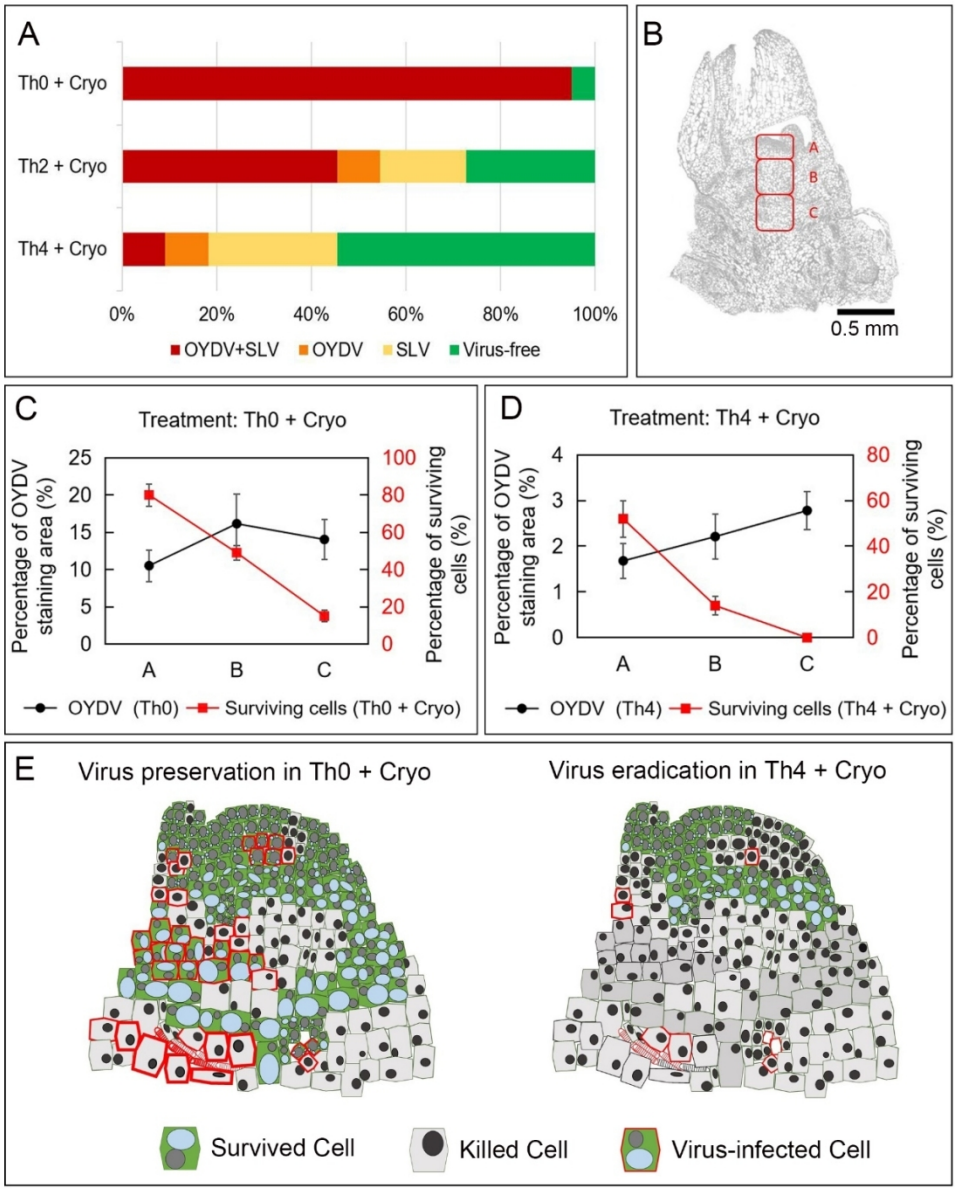
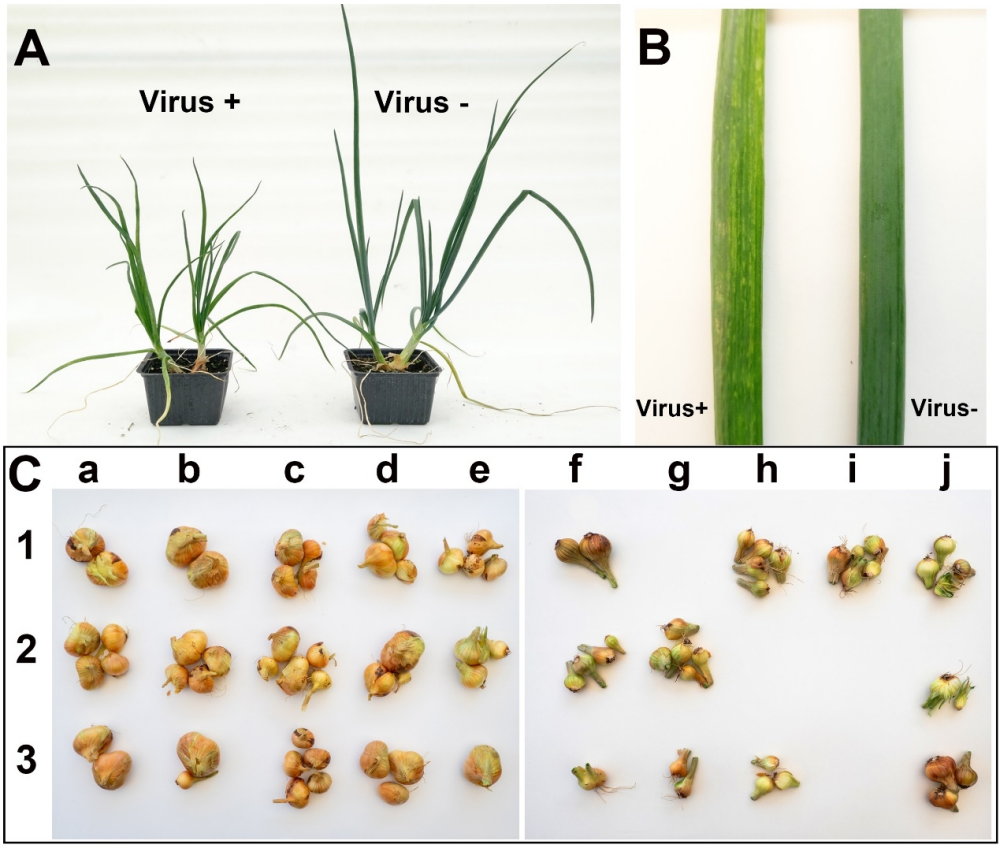


Figure 5 Combining thermotherapy with cryotherapy for preservation and eradication of viruses. (A) Percentage of various virus infection status in plants after combining thermotherapy with cryopreservation. (B) Three designated areas 'A', 'B' and 'C' in the sections of shoot tip used for measurement of OYDV signals and survived cells. (C) Percentages of areas stained from the localization of OYDV in the shoot tips before thermotherapy (Th0) and percentages of surviving cells after the following cryotherapy (Th0+Cryo) in three designated zones. (D) Percentages of areas stained from the localization of OYDV in the shoot tips after 4 weeks of thermotherapy (Th4) and percentages of surviving cells after the following cryotherapy (Th4+Cryo) in three designated zones. (E) Schematic illustration of cryopreservation (Th0+Cryo) and combining 4 weeks of thermotherapy with cryopreservation (Th4+Cryo) for virus elimination. Data represent the means ± SE of ten samples.

146x183mm (220 x 220 DPI)



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146x125mm (220 x 220 DPI)

Table 1 Effects of combining thermotherapy with shoot tip culture (STC) or cryotherapy (Cryo) on plant regrowth and elimination of onion yellow dwarf virus (OYDV) and shallot latent virus (SLV) from shallot '10603' plants

Thermotherapy (weeks)	Shoot regrowth (%)[a]		Virus-free efficiency (%)[b]					
			OYDV		SLV		OYDV + SLV	
	STC	Cryo	STC	Cryo	STC	Cryo	STC	Cryo
0	100w	55 ± 4y	0 (0/20)	5 (1/20)	0 (0/20)	5 (1/20)	0 (0/20)	5 (1/20)
2	100w	48 ± 4y	0 (0/15)	45 (5/11)	0 (0/15)	36 (4/11)	0 (0/15)	27 (3/11)
4	76 ± 6x	32 ± 2z	0 (0/15)	82 (9/11)	0 (0/15)	64 (7/11)	0 (0/15)	54 (6/11)

[a] shoot tips used were in the size of 2-3 mm and data are presented as means ± SE. Different letters in the same column indicate significant differences analyzed by one-way ANOVA with Tukey's test at $P < 0.05$.

[b] Numbers in parentheses are plantlets showing negative reaction to the virus / total samples analyzed by reverse-transcription polymerase chain reaction.

Table 2 Comparison of vegetative growth and bulb production between virus-infected and virus-free shallot '10603' plants

	Leaf number	Longest leaf length (cm)	Number of shoots/ plant	Total Bulb weight (g)	Bulb number
Virus-infected	11.3 ± 1.1 b	32.4 ± 1.3 b	1.9 ± 0.2 a	22.0 ± 2.9 b	2.5 ± 0.3 b
Virus-free	17.2 ± 0.8 a	44.3 ± 1.1 a	2.3 ± 0.2 a	54.8 ± 2.3 a	3.4 ± 0.2 a

Data were presented as means ± SE and with different letters in the same column indicate significant differences at $P < 0.05$ by Student's *t* test (n=21)