
Combining thermotherapy with meristem culture for improved eradication of onion yellow dwarf virus and shallot latent virus from infected *in vitro*-cultured shallot shoots

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Abstract

The present study described a combining thermotherapy with meristem culture for improved eradication of onion yellow dwarf virus (OYDV) and shallot latent virus (SLV) from co-infected *in vitro*-cultured shallot shoots. *In vitro*-cultured shoots infected with OYDV and SLV were thermo-treated at a constant temperature of 36 °C for 0, 2 and 4 weeks, and then meristems (0.5 mm) containing 1-2 leaf primordia were excised and cultured for shoot regrowth. Meristem culture without thermotherapy produced much higher levels of survival (of the 100%) and shoot regrowth (of the 55%) than those (62% survival and 32% shoot regrowth) produced by 4 weeks of thermotherapy and meristem culture. However, much higher virus-free frequencies (of the 70% for OYSV, 80% for SLV, and 50% for both viruses) were obtained in the former than those (of the 10% for OYSV, 15% for SLV and 10% for both viruses) obtained in the former. Histological and subcellular studies showed that thermotherapy imposed stress or damage to the cells of meristems, thus resulting in reduced meristem survival and shoot regrowth. Studies on virus location revealed considerable alternations of virus distribution patterns in the thermo-treated meristems. The results of histological and subcellular studies, and virus distribution provided explanations as to why combining thermotherapy with meristem culture improved the eradication of OYDV and SLV from the virus-infected *in vitro* shallot shoots.

Keywords: Meristem culture; onion yellow dwarf virus; shallot; shallot latent virus; thermotherapy;
ultrastructure; in vitro therapy..

1. Introduction

Shallot (*Allium cepa* var. *aggregatum*), a small-bulb onion crop, is widely grown in the subtropical and temperate regions of the world (Fritsch and Friesen, 2002). Shallot plants are propagated by bulbs, resulting in virus transmission from generation to generation and renders virus infection in commercially field-grown plants and germplasm collections (Smékalová et al., 2010; Katis et al., 2012). Viruses are among the most damaging pathogens that threaten the yield and quality of production of *Allium* species (Salomon, 2002； Katis et al., 2012). Onion yellow dwarf virus (OYDV, genus *Potyvirus*) and shallot latent virus (SLV, genus *Carlavirus*) are several viral pathogens of allium plants and prevail in many allium-growing regions of the worlds (Salomon, 2002; Conci et al., 2003； Smékalová et al., 2010; Shiboleth et al., 2010; Katis et al., 2012). Virus infection has been shown to reduce the vegetative growth, and bulb size and yield, and cause variety degeneration in *Allium* species (Conci et al., 2003; Perotto et al., 2010; Pramesh and Baranwal, 2015).

The use of virus-free propagating material has long been the most effective method for controlling virus diseases in vegetatively propagated plants (Salomon, 2002; Conci et al., 2005). Meristem culture (Walkey et al., 1987), combining thermotherapy with meristem culture (Walkey et al., 1987), combining chemotherapy with thermotherapy (Fletcher et al. 1998) and thermotherapy (Sulistio et al., 2015) were reported to eradicate OYDV and SLV from shallot. Combining thermotherapy with meristem culture were also found to eradicate OYDV and SLV from garlic (Conci and Nome, 1991; Perotto et al., 2003; Conci et al., 2005; Ramírez-Malagón et al., 2006). Continuous developments of efficient methods for the production

of virus-free plants would certainly assist in the sustainable production of *Allium* crops.

The objective of the present study was to test the efficacy of combining thermotherapy with meristem culture in eradicating of OYDV and SLV from co-infected *in vitro* shoots of shallots. Histological and subcellular alternations, and virus distribution patterns in the meristems without and with thermotherapy were analyzed to provide explanations as to why combining thermotherapy with meristem culture improves virus eradication frequency.

2. Materials and Methods

2.1. Plant materials, *in vitro* stock cultures and re-establishment of regenerated plants in greenhouse

Shallot genotype 10603 (*A. cepa* var. *aggregatum*) co-infected with OYDV and SLV was used in the present study. Bulbs were collected from greenhouse-grown plants that showed symptoms of yellow-green and chlorotic streaks on leaves (Fig. 1a) and used to establish the infected *in vitro* stock shoots, as described by Wang et al. (2019). The infected *in vitro* stock shoots were maintained on a shoot maintenance medium (SMM) composed of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose, 0.5 mg L^{-1} 6-benzylaminopurine (6-BA), 0.1 mg L^{-1} α-naphthylacetic acid (NAA) and 8 g L^{-1} agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 20 min. The stock cultures were maintained at 22 ± 2 °C under a 16-h photoperiod with a light intensity of 50 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes and subculture was done once every 4 weeks.

In vitro regenerated plantlets were tested by RT-PCR, as described below, for their virus status after 3 months of meristem culture without and with thermotherapy. The plantlets testing negative for both OYDV and SLV by RT-PCR were re-established in a net-proof greenhouse and grown for 5 months to produce bulbs. The bulbs were harvested and stored at 4 °C for 1 month. Then, the bulbs were grown in the same net-proof greenhouse. After two months of growth in the net-proof greenhouse, leaves were harvested and used for virus detection by RT-PCR.

2.2. RT-PCR assays

RT-PCR assays for OYDV and SLV were performed in the *in vitro* stock shoots prior to the virus eradication experiments, the *in vitro* –plantlets regenerated from meristem culture without and with thermotherapy, and the regenerated plants re-established in the greenhouse. The first time of virus tests was to confirm the virus status of the *in vitro* stock shoots before virus eradication experiments. The last two virus tests were used to assess the virus eradication frequencies after meristem culture following 0, 2 and 4 weeks of thermotherapy.

RT-PCR was conducted as described by MacKenzie et al. (1997) with some modifications. Total RNA was extracted from leaf tissue (0.5 g) using the Spectrum™ Plant Total RNA Kit (STRN250, Sigma, USA), according to the manufacturer's instructions. cDNA was synthesized using 2 µg of total RNA and the recombinant Moloney murine leukaemia virus reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The PCR reaction for OYDV and LSV was done in a 25 µL reaction volume containing 1 µL of each primer (10 µM), 0.5 µL of dNTPs (10 mM), 2 µL of template cDNA, 0.2 µL of *Taq* DNA

Polymerase with 2.5 µL of 10 x buffer (10342020, Invitrogen™, ThermoFisher Scientific, USA) and 17.8 µL of RNAase-free water. The primers for OYDV detection were as used by Mahmoud et al. (2008) and amplified a specific band of 601 bp of the coat protein gene (Table 1). The primers for SLV detection were designed using the software Primer premier 6.00 (PREMIER Biosoft International, CA, US) based on coat protein gene of garlic latent virus (GenBank: AB004565.1), to amply a specific band of 238 bp (Table 1).

2.3. Combining thermotherapy with meristem culture

Four-week old virus-infected *in vitro* stock shoots (Fig. 1b) were subjected to thermotherapy for 0, 2 and 4 weeks in a growth chamber set at a constant temperature of 36 ± 1oC under the same light conditions as those used for *in vitro* stock cultures. After thermotherapy treatments, meristems (0.5 mm in length) containing 1-2 leaf primordia (LPs, Fig. 1c) were excised from the infected *in vitro* shoots (Fig. 1d) and cultured at 24 ± 2oC on SMM in the dark for 3 days, and then cultured under the same conditions as those used for *in vitro* stock shoots for shoot regrowth. Subculture was performed once every 4 weeks.

2.4. Histological and subcellular studies

Meristems excised from the infected *in vitro* stock shoots following 0 and 4 weeks of thermotherapy were subjected to histological and subcellular studies. Histological analysis was performed as described by Wang et al. (2008). Briefly, the samples were fixed in FAA (50% ethanol: formalin: acetic acid; 18:1:1), dehydrated and embedded. Sections (5 µm) were cut

with a microtome (Leica DM 2235, Germany), stained with 0.1% toluidine blue (Sakai, 1973), and then examined under a light microscope (DM6 B, Leica, Wetzlar, Germany). Subcellular analysis was performed according to Wang et al. (2008). In brief, the samples were fixed, dehydrated and embedded in a low-viscosity embedding resin. Ultra-thin sections (70 μm) were cut with a microtome (Leica DM 2235, Germany) and stained with uranyl acetate and lead citrate. The grids were studied under a transmission electron microscope (Morgagni 268, FEI company B.V., The Netherlands). Cells locating down to 0.2 mm below the apical dome (AD) were analyzed and photographed.

2.5. Analysis of virus location and distribution patterns

Meristems were taken from the virus-infected *in vitro* stock shoots following 0 and 4 weeks of thermotherapy, and subjected to virus location and virus distribution pattern analysis. The virus was located as described by Wang et al. (2008). Briefly, samples were fixed in FAA, dehydrated and embedded. Thin paraffin sections (6 μm) were made using a rotary microtome (RM 2255, Leica, Nussloch, Germany). After the removal of paraffin by washing twice in xylene, the section were rehydrated, washed in PBS and treated with phosphate buffered saline (PBS) containing 4% bovine serum albumin for 30 min, and followed by overnight incubation at 5 ^{o}C with the rabbit polyclonal antibodies (Product No. 160212 for OYDV and 160812 for SLV, Bioreba, Switzerland), at a dilution of 1:500. After three rinses with PBS, the samples were incubated with anti-rabbit antibodies conjugated with alkaline phosphatase (A3937 Sigma Chemical Co., USA), at a dilution of 1:400 for 30 min at room temperature. After three rinses with PBS, the samples were stained using a freshly prepared

Fuchsin substrate solution. The sections were examined under a light microscope (DM6 B, Leica, Wetzlar, Germany).

For the analysis of virus distribution patterns in the meristem following 0 and 4 weeks of thermotherapy, the AD was divided into two zones. Tissue within a distance of 0.5 mm away from the AD was defined as Zone I and that beyond below 0.5 mm as Zone II. Fuchsin-stained purple reactions (virus signals of OYDV and SLV) that were seen closest to the AD, according to their positions within Zones I and II in each meristem, were marked and their number was counted

2.6. Experimental design and statistical analysis of data

In the experiment of virus eradication by meristem culture without and with thermotherapy, 30 meristems were included in each of the three treatments (0, 2 and 4 weeks of thermotherapy). All experiments were conducted twice. Data of meristem survival and shoot regrowth were presented as means ± standard error (SE) and analyzed using one-

directional ANOVA and student's *t*-test. Least significant differences (LSD) were calculated at $P<0.05$. Fifteen samples were used in each treatment of two replicates for virus location, analysis of virus distribution patterns, and histological and subcellular examination. Ten to 20 plants recovered from each of the virus eradication treatments, depending on the number of the plants available, were used for virus detection by RT-PCR to determine virus eradication frequencies.

3. Results

3.1 Effects of combining thermotherapy with meristem culture on meristem survival and shoot regrowth

Thermo-treated (4 weeks) *in vitro* stock shoots showed reduced growth and became yellowish in color, compared with non-heat-treated ones (Fig. 1d). Thermotherapy significantly affected survival and shoot regrowth of meristem culture (Table 2). All of the meristems without thermotherapy survived and 55% regenerated shoots. Levels of meristem survival and shoot regrowth markedly decreased as durations of thermotherapy increased from 0 to 4 weeks. As a result, 62% meristem survived and 32% regenerated shoots (Fig. 1e) in the meristems following 4 weeks of thermotherapy (Table 2).

3.2. Effects of combining thermotherapy with meristem culture on virus eradication

All in vitro stock shoots used for virus eradication experiments showed positive for both OYDV and SLV with RT-PCR, indicating the in vitro shock shoots were co-infected with OYDV and SLV (Fig. 2ab).Results of virus tests were consistent in the in vitro regenerants after 3 months of shoot regrowth and plants after 2 months of growth in the greenhouse. Therefore, results of virus tests by RT-PCR in the in vitro regenerated plant were presented. Virus-free frequency of meristem culture increased as duration of thermotherapy increased (Table 2). Meristem culture without thermotherapy resulted in 10% OYDV- and 15% SLV-free regenerated plant, and 10% were free of both OYDV and SLV. Meristem culture following 4 weeks of thermotherapy resulted in 70% OYDV- and 80% SLV-free regenerated plant, and 50% were free of both OYDV and SLV (Table 2).

3.4. Histological and subcellular examination

Histological studies showed that well-packed cells and densely stained cytoplasm containing nucleoli were observed in meristems without thermotherapy (Fig. 3a), indicating that cells were in a good physiological condition. In comparison, weakly stained cytoplasm containing densely stained nucleoli and looser tissues were found in thermo-treated meristems (Fig. 3b), indicating cells were stressed and damaged by thermotherapy.

Subcellular examination of the meristems without thermotherapy showed the meristematic cells were characteristic of dense cytoplasm, a

large nucleo-cytoplasm ratio, small vacuoles and various subcellular structures including mitochondria, plastids and endoplasmic reticulum (Fig. 3c). These characteristics indicated these cells were metabolically active cells. In contrast, two most distinct subcellular changes were observed in the thermo-treated meristems (Fig. 3d). The nucleo-cytoplasm ratio was reduced and the vacuoles were markedly enlarged (Fig. 3d), indicating signs of stress and damage to the cells brought about by thermotherapy.

3.5. Virus location and distribution patterns

When subjected to immuno-staining with antibodies against the coat proteins of OYDV and SLV, a purple color reaction (virus signals) develops in the virus-infected cells but not in the virus-free cells (Fig. 4a, b). In the meristems of the diseased *in vitro* shoots without thermotherapy, signals of OYDV and SLV were easily seen in the tissues across shoot tips including the AD and in the lower parts of shoot tips (Fig. 4c, d). Purple color reaction to OYDV and OYDV-infected area were much stronger and larger than SLV (Fig. 4c, d). OYDV was detected much closer to the AD than SLV (Fig. 4, d).

In our preliminary studies, we found virus distribution patterns varied greatly from meristem to meristem infected with the same virus. Therefore, we analyzed the virus distribution patterns in the 15 meristems without thermotherapy and 15 meristems following 4 weeks of thermotherapy. For meristems without thermotherapy, 12 and 11 out of 15 meristems tested showed OYDV and SVL in Zone I (Fig. 5a). In another word, 20% and 27% of the 0.5 mm meristems were free of OYDV and SLV infection (Fig. 5c). Four weeks of thermotherapy caused considerable alternations in virus distribution patterns, thus enlarging virus-free zones in the shoot tips (Fig.

4e, e1, f and f1). Analysis of virus distribution patterns found that 5 and 4 out of 15 meristems tested showed OYDV and SLV in Zone I (Fig. 5b). In another word, 66% and 73% of the 0.5 mm meristems were free of OYDV and SLV infection (Fig. 5c).

4. Discussion

Meristem culture was one of the major methods used for eradication of OYDV and SLV from the *Allium* plants including shallot and garlic. Using meristem culture (0.5-0.8 mm), Walkey et al. (1987) obtained 26% shallot plants and 25% garlic plants free of OYDV and SLV, respectively. Verbeek et al. (1995) obtained 91-100% OYDV-G-free frequencies of garlic plants in meristem culture (0.15-0.3 mm). Shiboleth et al. (2001) reported that meristem culture (0.2 mm) produced 62-65% garlic plants free of OYDV, *Leek yellow stripe virus* (LYSV) and *Garlic common latent virus* (GCLV). Recently, Vieira et al. (2015) obtained 26% and 40% OYDV-free plants regenerated from shoot tips (1–1.5 mm) and meristems (0.1-0.3 mm). In the present study of shallot, meristem culture with (0.5 mm in length with 1-2 LPs) resulted in 10% OYDV- and 15% SLV-free regenerated plants, and 10% were free of both OYDV and SLV. It is worth to note that Pramesh and Baranwal (2015) failed to obtain any OYDV- and SLV-free garlic plants, using meristem culture (with 0.5-1.0 mm) containing 1-2 leaf primordia. Virus-host combinations may cause the differences of virus eradication frequencies between Pramesh and Baranwal (2015) and the others (Walkey et al., 1987; Vieira et al., 2015 and the present study). However, specific reasons for this remained unknown yet.

Virus location in the meristems infected with a given virus provided valuable references used for measurement of a suitable meristem size for the eradication of the virus (Wang and Valkonen, 2008; Li et al., 2016; Wang et al., 2016; Zhao et al., 2018). *Apple stem grooving virus* (ASGV) was detected in part of the tissue across the AD of the meristem, while *Apple stem pitting virus* (ASPV) was not detected in AD but only in the lower part of the meristem of the apple rootstock 'M9' (Li et al., 2016) and scion 'Gala' (Wang et al., 2016). Although ASGV was detected in the AD of the meristems in the two apple cultivars 'Gala' and 'Ruixue', a few top layer cells of the AD were free of ASGV infection, forming a virus-free area. The virus-free area appeared to be larger in 'Ruixue' than in 'Gala', indicating the different infectious ability of ASGV to invade the AD of the two apple cultivars (Zhao et al., 2018). The present study found although the two viruses were present in the meristem, the area free of SLV in the AD was larger than that of OYDV, and SLV was detected in the lower part of the AD than OYDV. These data explained why SLV-free frequency was higher (15%) than that of OYDV (10%) in meristem culture without thermotherapy. Analysis of the virus distribution patterns found that 20% and 27% of 0.5 mm meristem were free of OYDV and SLV infection, respectively. These figures appeared to be greater than the virus-free frequencies. The reason for this might be that meristem sizes that were actually taken were either smaller or larger than 0.5 mm, because precise excision of a designed size of meristems by hands is difficult.

Combining thermotherapy with meristem culture has been well-documented to improve virus eradication in various plants including *Allium crops*, but these treatments reducing meristem survival and shoot regrowth in various plants including *Allium* crops (Wang et al., 2018).

Ramírez-Malagón et al. (2006) showed in two garlic cultivars that 34-42% of meristems without thermotherapy survived, while 27%-37% of the thermo-treated meristems survived. Similar results were also reported in garlic 'Jonas' by Vieira et al. (2015). Conci and Nome (1991) reported that 82% of meristems without thermotherapy regenerated shoots, while only 57% of meristems following thermotherapy (36 ºC for 40 days) produced shoot regrowth in five garlic cultivars. Reduced levels of shoot regrowth in the thermo-treated meristems were repeatedly reported in *Allium* crops (Robert et al., 1998, Conci et al., 2005, Perotto et al., 2003, Pramesh and Baranwal, 2015). All of the above results agreed with our results. The present study further found that thermotherapy for 4 weeks induced formation of looser tissue, reduced nucleo-cytoplasm ratio and increased vacuole size in the meristems. Similar results were obtained in thermo-treated raspberry shoot tips (Wang et al., 2008) and garlic shoot tips (Vieira et al., 2015). These histological and subcellular alternations indicated that thermotherapy induced stress or damage to the cells, thus resulting in the reduced meristem survival and shoot regrowth.

Meristem culture alone produced 25% OYDV- and SLV-free plants, which were much lower than 84% virus-free plants obtained by thermotherapy (38 ºC for 34 days) followed by meristem culture in two garlic cultivars (Walkey et al., 1987). Conci and Nome (1991) found that meristem culture alone resulted in 11% OYDV- and SLV-free plants, and thermotherapy (36 ºC for 40 days) followed by meristem culture increased the virus-free frequency to 62% in five garlic cultivars. Similar results were reported in a number of studies using thermotherapy followed by meristem culture for eradication of OYDV and SLV from the infected garlic plants (Robert et al., 1998; Senula

et al., 2000; Perotto et al., 2003, Ramírez-Malagón et al., 2006; Pramesh and Baranwal, 2015; Sulistio et al., 2015; Vieira et al., 2015). All these data were supported by the results obtained in the present study.

There have been several studies that attempted to elucidate the mechanism why combining thermotherapy with meristem culture improved virus eradication. Virus location showed that thermotherapy inhibited movement of the virus toward the AD of the meristem, thus enlarging the virus-free areas in the meristems in raspberry infected with raspberry bushy dwarf virus (RBDV, Wang et al., 2008) and apple infected with ASGV (Zhao et al., 2018). Results of virus location and analysis of virus distribution patterns reported in the present study were consistent with those of Wang et al. (2008) and Zhao et al. (2018). Following 4 weeks of thermotherapy, 67% and 73% of 0.5 mm meristems were found free of OYDV and SLV, respectively, which were close to 70% OYDV- and 80% SLV-free frequencies obtained in the thermo-treated meristems (0.5 mm). Thermotherapy was also reported to inhibit viral replication (Wang et al., 2008; Zhao et al., 2018), cause virus RNA degradation (Wang et al., 2008; Zhao et al., 2018), and induce virus-induced RNA silencing (Wang et al., 2008; Liu et al., 2015, 2016). All these effects of thermotherapy on virus may eventually lead to improved virus eradication by combining thermotherapy with meristem culture.

In conclusion, combining thermotherapy with meristem culture was described for improved eradication of OYDV and SLV from the infected *in vitro* shallot shoots. Histological and subcellular examination provided explanations for the reduced meristem survival and shoot regrowth in thermo-treated meristems. Virus location and analysis of virus distribution patterns explained why OYDV and SLV eradication frequencies differed each other in the same method and revealed why combining thermotherapy

with meristem culture largely improved OYDV and SLV eradication. The combining thermotherapy with meristem culture described in the present study would provide technical supports for the production of virus-free shallot stockplants.

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Conflicts of interest

The authors declare no potential conflict of interest

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