

ORIGINAL ARTICLE

Comparison of three different techniques for eradication of *Apple mosaic virus (ApMV)* from hazelnut (*Corylus avellana* L.)

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Abstract

Numerous plant species around the world suffer from the presence of viruses, which especially in economically important crops, cause irretrievable damage and/or extensive losses. Many biotechnological approaches have been developed, such as meristem culture, chemotherapy, thermotherapy or cryotherapy, to eliminate viruses from infected plants. These have been used alone or in combination. In this work, meristem culture, thermotherapy and cryotherapy were compared for *Apple mosaic virus* elimination from hazelnut local cultivar “Palaz”. The virus-free plant was also confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) after each treatment and, the best results were obtained by cryotherapy. A one step freezing technique, droplet vitrification, was used for cryotherapy, and the best regeneration percentage was 52%. After cryotherapy, virus-free seedlings of hazelnut local cultivar “Palaz” were confirmed as being virus-free after three subcultured periods.

Keywords: cryotherapy, droplet vitrification, meristem culture, PVS2, RT-PCR, thermotherapy

Introduction

Plant viruses are major pathogens that cause economic losses and damage for many crops, fruits, vegetables, and woody plants. Nearly all plants are influenced by at least one virus (Gergerich and Dolja 2006). Because viruses cause great economic losses and destruction in plants, they have been the subject of much research (Milosevic *et al.* 2012).

The Black Sea region of Turkey which is the gene origin of hazelnut (*Corylus avellana* L.) is the most important area of the world for hazelnut production. However, this production is limited by *Apple mosaic virus* (ApMV) belonging to Bromoviridae family and *Illarvirus* genus (Akbas and Degirmenci 2009). The ApMV causes different symptoms such as leaf spots, yellow ringspots and oak leaf motifs. These are obvious during growth and they are partially latent during stationary phases of hazelnut trees. Their transmission from one tree to another occurs by root bridges. The most harmful effects of ApMV are the reduction of hazelnut fruit size and yield (Kobylko *et al.* 2005).

Plant viruses can be controlled by quarantine, isolation, sanitation and certification programs depending on sensitive and specific methods. Biotechnological approaches including meristem culture, thermotherapy and cryotherapy provide the most effective ways of obtaining virus-free plants and establishing virus-free plantations (Nukari *et al.* 2009; Wang *et al.* 2009). Meristem culture which is applied to various plant species uses tissue culture technology. The basic principle of this technique is excision of 0.1–0.7 mm meristem (depending on explant type) and regeneration on semi-solid media supplemented with plant growth regulators. In this way, the plants regenerated from meristematic domes are free from viruses and other pathogens (Slack and Tufford 1995).

In thermotherapy, whole plants or different types of explants infected by viruses are kept at high temperatures (35 to 54°C, depending on the plant's physiological tolerance) for suitable periods of time. The basic principle of this method is to find which temperature and

treatment time is the best for virus reduction and the survival of the whole or part of a plant. Since thermal sensitivity of some plant cells or tissues is higher than some viruses, the plant tissues damaged by thermal stress can regenerate more rapidly than viruses (Spiegel *et al.* 1993).

Cryotherapy has been effectively applied for virus elimination from different kinds of plant species such as potato, banana, grape, and strawberry (Wang and Valkonen 2009). The main principle of this technique is that explants (meristems, shoot tips, nodal or apical buds, embryos) are frozen rapidly in liquid nitrogen (-196°C) after physical dehydration or chemical vitrification for at least 24 h. Then the explants are transferred to a previously optimized regeneration media. This is a newly developed technique, and in the literature, there are few studies which compare cryotherapy with other traditional virus elimination techniques. To date, results concerning virus-free plant verification and acclimatization to greenhouse conditions after cryotherapy treatments are still not available (Feng *et al.* 2013; Wang *et al.* 2014; Bettoni *et al.* 2016; Kaya *et al.* 2020).

Biotechnological developments have played a crucial role for fast, sensitive and specific determination

of plant viruses. In recent years, protocols based on polymerase chain reaction (PCR) performed with specific nucleic acid sequences such as virus capsid protein genes, have been effectively used for diagnostic trials of infected plants (O'Donnell 1999; Ward *et al.* 2004). Reverse transcriptase (RT)-PCR has been used to achieve highly specific and more sensitive trials for the determination of some RNA viruses, including ApMV virus (Valasevich *et al.* 2014).

For this reason, the first aim of the current work was to compare the efficiency of cryotherapy with meristem culture and thermotherapy techniques for virus elimination from *C. avellana* L. cv. "Palaz" infected with *Apple mosaic virus*. The second aim was to confirm virus free plants using reverse transcription polymerase chain reactions (RT-PCR).

Materials and Methods

Plant material

Symptomatically infected hazelnut local cultivar "Palaz" shoots having leaves with pale yellow to bright cream colored stains (Fig. 1A–B) and uninfected

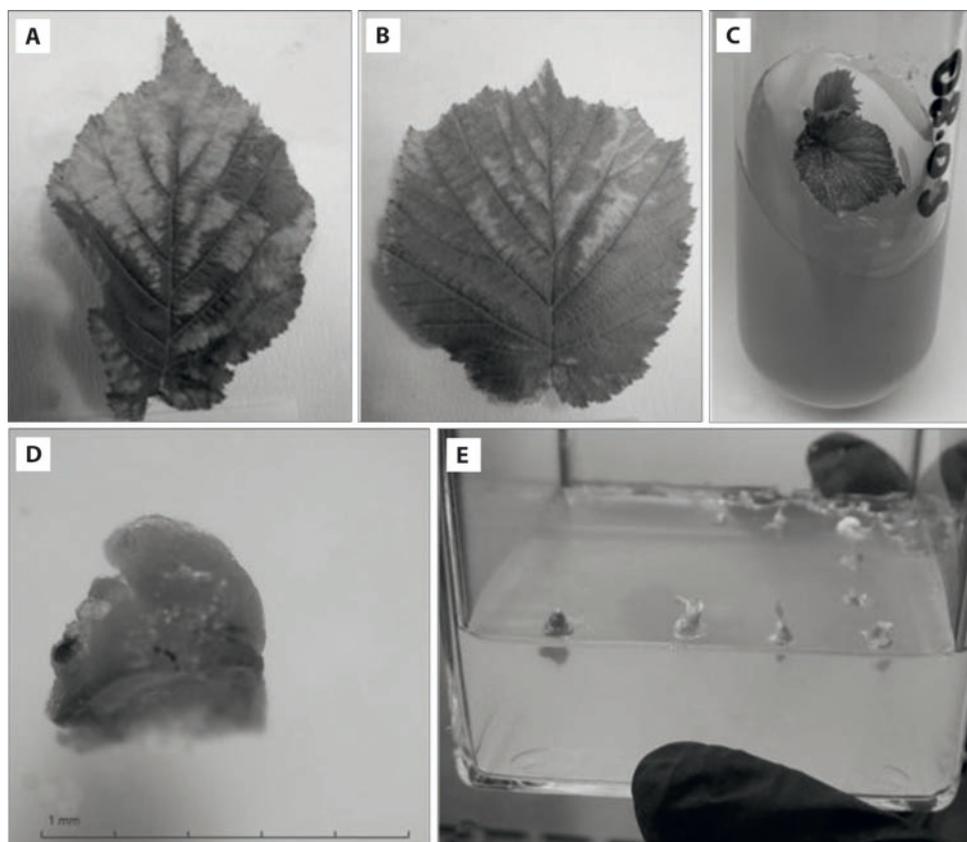


Fig. 1. Symptomatically infected leaves of *Corylus avellana* local cultivar "Palaz" with pale yellow to bright cream colored stains (A, B); *in vitro* grown shoots (C) and excised meristem (D) from *in vitro* grown shoots of infected hazelnut local cultivar "Palaz"; meristem regeneration in Magenta GA-7 vessels including woody plant medium (WPM) supplemented with $4.44 \mu\text{mol} \cdot \text{l}^{-1}$ benzyl adenine (BA), $10 \text{ mg} \cdot \text{l}^{-1}$ FeEDDHA and $30 \text{ g} \cdot \text{l}^{-1}$ sucrose

shoots (for control) were obtained from the Hazelnut Research Station, Giresun–Turkey.

Surface sterilization and *in vitro* propagation of hazelnut shoot tips

Hazelnut shoot tips (~1 cm long) belonging to “Palaz” cultivar were surface sterilized by treating for 5 min with 70% ethanol and two times for 10 min with 10% concentrated commercial bleach, Domestos®. After each step, the shoots were rinsed in sterile distilled H₂O consecutively (Ozudogru *et al.* 2011). After surface sterilization, the lower, brownish sides of cut shoots were directly transferred to semi-solid regeneration medium [woody plant medium (WPM, Lloyd and McCown 1980) supplemented with 4.44 μmol · l⁻¹ benzyl adenine (BA), 10 mg · l⁻¹ FeEDDHA and 30 g · l⁻¹ sucrose] under standard culture conditions, 27 ± 2°C, 16/8 photoperiod with 50 μmol⁻¹ · m⁻² · s⁻¹ cool daylight fluorescent lamps.

Meristem culture

The meristems (~0.3–0.5 mm) were excised from *in vitro* grown hazelnut shoot tips of cv. “Palaz” infected with ApMV (Fig. 1C–D) under a microscope and directly transferred to Petri dishes with semi-solid regeneration medium described above. They were incubated in the dark at 27 ± 2°C for 48 h, and then they were transferred to standard culture conditions described above during the regeneration period. When the meristems started to regenerate (after 5–7 days), they were transferred to Magenta GA-7 vessels including regeneration medium (Fig. 1E).

Thermotherapy treatments

In vitro grown *C. avellana* shoots (Fig. 2A) were transferred to a growth chamber (25°C) for 48 h (16/8 h photoperiods) and, every 24 h, the temperature was raised 1°C till the final temperature was 40°C (15 days). The plants were maintained for 3 more weeks under these conditions. Then the meristems were excised from the

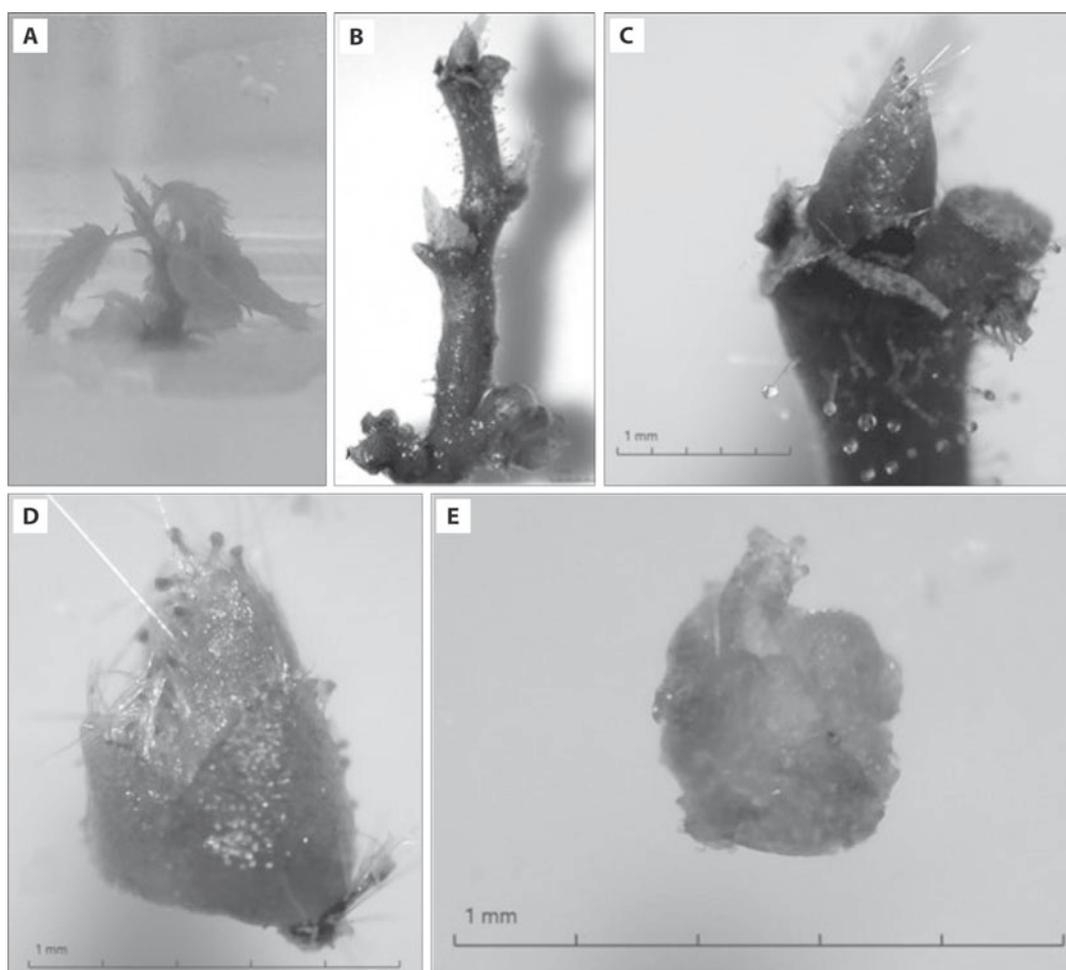


Fig. 2. *In vitro* grown *Corylus avellana* cv. “Palaz” shoot (before thermotherapy treatment): A – after thermotherapy, B – shoot, C – shoot apex, D – shoot tips, E – meristem of *C. avellana* cv. “Palaz”

shoots treated with thermotherapy conditions (Díaz-Barrita *et al.* 2008; Vivek and Modgil 2018; Fig. 2B–E). The meristems were transferred to Petri dishes including regeneration medium (previously described) supplemented with $10 \text{ mg} \cdot \text{l}^{-1}$ charcoal under dark conditions at 25°C for 48 h. Then, they were transferred to standard culture conditions, $27 \pm 2^\circ\text{C}$, 16/8 photoperiod with $50 \mu\text{mol}^{-1} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ cool daylight fluorescent lamps.

Cryotherapy of *Corylus avellana* meristems

The meristems were excised from *in vitro* grown *C. avellana* cv. “Palaz” shoots infected with ApMV (the shoots were cold hardened for 2 weeks in the dark at $+4^\circ\text{C}$, Fig. 3A–B) and, for sucrose preculture, they were transferred to WPM medium supplemented with $4.44 \mu\text{mol} \cdot \text{l}^{-1}$ BA and 0.4 M sucrose for 24 h (Kaya *et al.* 2013; Kaya and Souza 2017; Fig. 3C). After sucrose preculture, meristems were cryotherapied via droplet vitrification based on PVS2 chemical vitrification [30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) supplemented with 0.4 M sucrose in liquid medium (Sakai *et al.* 1990)]. In this technique, the meristems were treated with $3 \mu\text{l}$ PVS2 dropped on a $3 \times 12 \text{ mm}$ aluminium foil strip (Fig. 3D) at different exposure times (15, 30, 45, 60, 75, 90 min) and then plunged directly into

liquid nitrogen (LN). Control groups were directly transferred to semi solid regeneration medium following washing (to remove PVS2) with liquid WPM medium supplemented with 1M sucrose. After at least 24 h exposure to LN, the samples were thawed by washing solution, then the meristems were placed in Petri dishes including semi solid regeneration media in dark at $27 \pm 2^\circ\text{C}$ for 48 h. After 48 h the meristems were transferred to the standard culture described above. When the meristems began to regenerate (approximately 10 days later), they were transferred to Magenta GA-7 vessels with fresh semi solid regeneration medium.

Evaluation of data and statistical analyses

For each treatment of meristem culture, thermotherapy and cryotherapy, 20 meristems (10 for controls, 10 for main treatments) were used. All treatments were repeated at least three times. The data of *in vitro* regeneration of hazelnut meristems (untreated, treated with thermotherapy and cryotherapy) were collected after 4 weeks (untreated meristems, after thermotherapy and control groups of cryotherapy) and 6–8 weeks (the meristems exposed to liquid nitrogen) after incubation on regeneration medium. At least one elongated shoot derived from meristems was considered as a successful regeneration for each treatment. The collected data

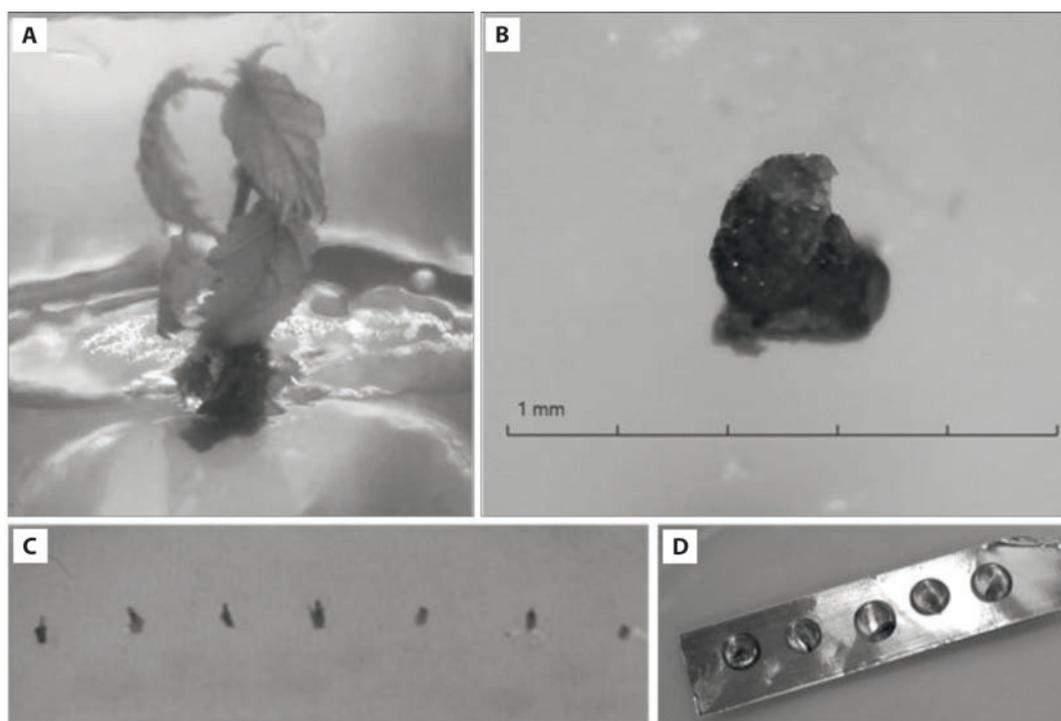


Fig. 3. The shoot of hazelnut cv. “Palaz”, after 2 weeks of cold hardening in the dark at $+4^\circ\text{C}$ – A; the excised meristem from cold hardened shoot – B; the meristems after 24 h sucrose preculturing – C; the meristems treated with $3 \mu\text{l}$ drops of PVS2 for cryotherapy via droplet vitrification technique

were calculated as regeneration rate, shoot number derived from each meristem and, shoot length. The shoot forming capacity (SFC) index was calculated according to the following formula (Lambardi *et al.* 1993):

$$\text{SFC index} = [(\text{average shoot number obtained per regenerated meristem}) \times (\text{regeneration percentage})] / 100.$$

The regeneration percentage and obtained shoot numbers were compared by multiple X^2 test by SPSS statistics program (IBM SPSS Statistics 21.0) and statistical analysis was performed by ANOVA, followed by the LSD test at $p \leq 0.05$ (Marascuilo and McSweeney 1977).

RNA isolation and reverse transcriptase polymerase chain reactions (RT-PCR)

Lithium chloride-based protocol was used for total RNA extractions from the samples derived from hazelnut mother plants (non-infected and infected with ApMV), meristem culture and cryotherapy treated meristems (Spiegel *et al.* 1996). One step RT-PCR reactions were performed using two oligonucleotide primers (reverse, 5'-ATC CGA GTG AAC AGT CTA TCC TCT AA-3'; forward, 3'-GTA ACT CAC TCG TTA TCA CGT ACA A-5', primer position 1474–1499, 1711–1735; product size 262 bp, accession number U15608; Menzel *et al.* 2002). These primers were used to detect the strain of the virus. The PCR reactions were performed in 25 μl of reaction mix containing 50 ng RNA template, 20 pmol $\cdot \mu\text{l}^{-1}$ of each primer, 0,4 mM dNTP mix, 20 unit reverse transcriptase,

1 unit of Taq DNA polymerase, 12 u $\cdot \mu\text{l}^{-1}$ RNase inhibitor, 2,5 μl 1x reaction buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], and 1.5 mM MgCl_2 (Sellner *et al.* 1992). The cDNA amplification was achieved by incubation of the reaction mixture at 42°C for 30 min, then the reaction was continued as follows: 3 min at 95°C, followed by 40 cycles of 30 s at 92°C, 30 s at 54°C and, 1 min at 72°C with a final extension of 5 min at 72°C. PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide for visualising (Kaya 2015).

Results

Meristem culture of infected hazelnut cv. "Palaz"

The regeneration percentages of all *C. avellana* cv. "Palaz" meristems were obtained as 100 and shoot forming capacity index was calculated as 1.7 (Table 1). All shoots derived from meristem culture were well formed after three subculturing periods. On the leaves of these shoots there were pale yellow or cream colored stains, characteristic for ApMV infections.

Thermotherapy for obtaining virus-free hazelnut

The meristems of *C. avellana* cv. "Palaz" treated with thermotherapy did not regenerate (Table 1). After 2 days of incubation, they turned brown because they died.

Table 1. Regeneration percentages and calculated shoot forming capacity index of *Corylus avellana* cv. "Palaz" after meristem culture, thermotherapy and cryotherapy treatments (SE – standard error)

Treatment	Regeneration rate [% \pm SE]	Mean shoot number [no. \pm SE]	Mean shoot length [mm \pm SE]	Shoot-forming capacity (SFC) index	
Meristem culture treatments	100 \pm 0.0 a	1.7 \pm 0.3 a	7.2 \pm 1.1 a	1.7	
Thermotherapy treatments	0	0	0	0	
Cryotherapy treatments (control groups)	30 min PVS2	86.7 \pm 1.7 b	1.3 \pm 0.2 a	5.0 \pm 0.6 cd	1.1
	45 min PVS2	86.7 \pm 3.7 b	1.0 \pm 0.0 b	6.3 \pm 0.7 ab	0.9
	60 min PVS2	80 \pm 0.0 c	1.3 \pm 0.1 a	5.5 \pm 0.3 bc	1.0
	75 min PVS2	83.3 \pm 2.3 bc	1.0 \pm 0.0 b	5.0 \pm 0.4 cd	0.8
	90 min PVS2	73.3 \pm 2.8 d	1.0 \pm 0.0 b	5.0 \pm 0.6 cd	0.7
Cryotherapy treatments (liquid nitrogen groups)	30 min PVS2	0	0	0	0
	45 min PVS2	23.3 \pm 0.9 f	1.0 \pm 0.0 b	2.7 \pm 0.1 e	0.2
	60 min PVS2	46.7 \pm 0.9 e	1.3 \pm 0.1 a	4.3 \pm 0.1 d	0.6
	75 min PVS2	6.7 \pm 0.8 g	0.7 \pm 0.1 c	1.7 \pm 0.2 f	0.1
	90 min PVS2	0	0	0	0

Percentage values statistically analyzed by a non-parametric test, the post hoc multiple comparisons test (Marascuilo and McSweeney 1977). Small letters indicated that the values have homology according to statistical analysis performed by ANOVA, followed by LSD test at $p \leq 0.05$

Cryotherapy via droplet vitrification

The best regeneration percentage of cryotheraped meristems of *C. avellana* cv. "Palaz" was 46.7% obtained from samples dehydrated with PVS2 for 60 min. The calculated shoot forming capacity index was 0.6 (Table 1). After cryotherapy treatments, the shoot apices exposed to liquid nitrogen developed well formed shoots, and there were no pale yellow or cream colored stains indicating ApMV infection on their leaves after initiation of regeneration and also after three subculturing periods.

Confirmation of virus-free plants via RT-PCR

Excised meristems obtained from *in vitro* grown ApMV infected *C. avellana* cv. "Palaz" shoots produced well formed shoots and they had no symptomatic signs of ApMV infection. However, RT-PCR results showed that all of the shoots derived from meristem culture were infected with ApMV (Fig. 4). On the other hand, the shoots derived from cryotheraped meristems had no virus infections according to RT-PCR results when they were compared with mother plants and initiation cultures (Fig. 5).

Comparison of three virus elimination methods

The results of the current study showed that the best method was cryotherapy for virus elimination of *C. avellana* cv. "Palaz" infected with ApMV. On the other hand, the results of meristem culture seemed to be efficient methods for virus elimination, but the RT-PCR results showed that the ApMV infection was

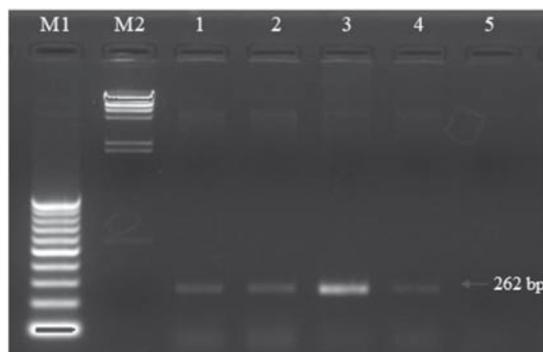


Fig. 4. Agarose gel electrophoresis of RT-PCR products. The total RNA sources of: 1 – mother plant symptomatically infected with ApMV; 2 – *in vitro* grown shoots derived from infected shoot tips; 3 – after initial regeneration of meristem culturing; 4 – after three subculturing periods of meristem culturing; 5 – symptomatically uninfected plant (negative control) of *Corylus avellana* cv. "Palaz"; M1 – 100 bp ladder molecular size marker; M2 – Lambda/*Hind*III Marker

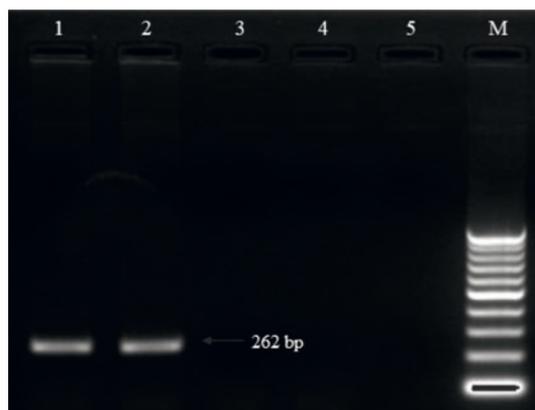


Fig. 5. Agarose gel electrophoresis of RT-PCR products. The total RNA sources of: 1 – mother plant symptomatically infected with ApMV; 2 – *in vitro* grown shoots derived from infected shoot tips; 3 – after initial regeneration of cryotheraped meristems; 4 – after three subculturing periods of cryotheraped meristems; 5 – symptomatically uninfected plant (negative control) of *Corylus avellana* cv. "Palaz"; M – 100 bp ladder molecular size marker

still present in hazelnut shoots. Thermotherapy was not successful for virus elimination, because no living or regenerating shoots were obtained (Table 1).

Discussion

Plant viruses can cause a lot of epidemics in different wild-type and/or economical important crops in the world. These epidemics can also cause significant economic losses. For this reason, the development of sensitive technologies for characterization and identification of plant viruses plays an important role in eradicating them from plants (Milosevic *et al.* 2012). Several methods have been developed to eradicate viruses from infected plants including chemotherapy, meristem culture, thermotherapy and cryotherapy and have been used alone or in combination with other methods (Papstein *et al.* 2013; Hu *et al.* 2015). In this work, the efficiency of meristem culture, thermotherapy and cryotherapy was compared for eradication of ApMV from infected *C. avellana* cv. "Palaz". Hu *et al.* (2015) used thermotherapy and chemotherapy for the elimination of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), and *Apple stem pitting virus* (ASPV) from apple. Balamuralikrishnan *et al.* (2002) investigated combined effects of meristem culture and chemotherapy for eradicating *Sugarcane mosaic virus* (SCMV) from infected *Saccharum officinarum* L. Helliot *et al.* (2002) used cryotherapy for the eradication of *Cucumber mosaic virus* (CMV) and *Banana streak virus* (BSV) from *Musa* spp.

Meristem culture is an efficient tool for eradicating viruses from infected plants. It has been widely used to obtain virus-free plants of different plant species (Rout *et al.* 2006). Ramgareeb *et al.* (2010) obtained virus-free sugarcane using meristem culture from plants infected with *Sugarcane mosaic virus* and *Sugarcane yellow leaf virus*. Kumar *et al.* (2009) used meristem culture for the elimination of *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) from *Chrysanthemum morifolium* Ramat. cv. Pooja plants. In this work meristem culture was used to eliminate ApMV virus from infected *C. avellana* cv. "Palaz". Symptomatically healthy shoots were obtained by using this procedure, however, RT-PCR results showed that the shoots still had ApMV infection. This may have been caused by the usage of ~ 0.3–0.5 mm (depending on explant type) meristems of *in vitro* grown hazelnut shoots. This size range (~ 0.3–0.5 mm) can not be enough for virus elimination, but the hazelnut shoot tips of cv. "Palaz" were too large to excise meristems smaller than 3 mm (Fig. 3C–D). Ramgareeb *et al.* (2010) obtained virus-free plants from only 2 mm or a smaller sized meristem of sugarcane shoots. Also, Kumar *et al.* (2009) used between 0.25–0.3 mm meristems of *Ch. morifolium* Ramat. cv. Pooja shoots for obtaining virus-free plants.

In this study thermotherapy was combined with meristem culture. However, excised meristems from thermotherapied shoots of *C. avellana* cv. "Palaz" did not regenerate. This may have been caused by the thermal sensitivity of *C. avellana*. During thermotherapy treatments, the hazelnut shoots were exposed to high thermal conditions (up to 40°C). The hazelnut temperature conditions were determined as being between 13 and 16°C i.e. the annual mean temperature limits in hazelnut farming areas (Ustaoglu and Karaca 2010). However, in *in vivo* and *in vitro* grown plants, thermotherapy treatments can reduce virus concentration and increase the efficiency of virus elimination (Tan *et al.* 2010). Papstein *et al.* (2008) used thermotherapy to obtain virus-free plants from apple cultivars infected with *Apple chlorotic leaf spot virus* (ACLSV) and with *Apple stem pitting virus* (ASPV). López-Delgado *et al.* (2004) used a modified procedure of standard thermotherapy to eradicate *Potato virus X* (PVX) from infected *in vitro* grown *Solanum tuberosum*.

In this study, the droplet vitrification technique was used to obtain virus-free plants from infected *C. avellana* cv. "Palaz" shoots. This cryotherapy treatment was combined with meristem culture. Wang and Valkonen (2008a) used shoot tip culture and cryotherapy to obtain virus-free plants from sweet potato (*Ipomoea batatas*) infected with *Sweet potato chlorotic stunt virus* (SPCSV; Closteroviridae) and *Sweet potato feathery mottle virus* (SPFMV; Potyviridae). Their

results showed that shoot tip culture was effective for only shoot tips of 1 mm or less in size for sweet potato. However, in cryotherapy, shoot tips larger than 1 mm in size could be used for efficient virus elimination from infected sweet potato. This was because the apical dome and only two of the youngest leaf primordia can survive after liquid nitrogen exposure during cryotherapy (Wang *et al.* 2008; Wang and Valkonen 2008b). Wang *et al.* (2006) used meristem culture, thermotherapy and cryotherapy to obtain virus free plants from *in vitro* grown potato shoot tips infected with *Potato leafroll virus* (PLRV) and *Potato virus Y* (PVY). Their results showed that cryotherapy could be an efficient method for the elimination of potato viruses when comparing meristem culture and thermotherapy. Similar results were obtained from the current study. After cryotherapy, all regenerated meristems of *C. avellana* cv. "Palaz" derived from ApMV infected shoots were virus-free when they were confirmed by RT-PCR (Fig. 5).

Fast, reliable and cheap procedures for the determination of viruses from infected plants can play an important role in routine work, therefore, PCR based procedures can provide an alternative way to effective diagnosis. Hu *et al.* (1995) compared three methods, dot blot hybridization, enzyme-linked immunosorbent assay (ELISA) and RT-PCR, to detect two *Cucumber mosaic viruses* in infected banana plants. They found that the RT-PCR was a more sensitive tool than either dot blot hybridization or ELISA. Therefore, in the current work RT-PCR was used to detect ApMV for all samples.

Conclusions

In this study three different virus elimination protocol for obtaining virus-free plants of *C. avellana* cv. "Palaz" infected with ApMV were compared. The cryotherapy method based on chemical vitrification and one step freezing protocols was effective for obtaining virus-free *C. avellana* cv. "Palaz". In conclusion, cryotherapy can be a useful tool for future studies on different wild-type and culture species of *Corylus* genus. At the same time, confirmation of virus plants using molecular methods such as RT-PCR can be effective, reliable and fast techniques for detection of viruses from infected plants.

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