Phytoparasitica (2023) 51:855–864
https://doi.org/10.1007/s12600-023-01101-x

RESEARCH

European and American grapevines were successfully recovered from GFkV, GLRaV1, GLRaV2, and GLRaV3 viruses by a modified thermotherapy and shoot tip culture

Hayri Sağlam · Özlem Çalkan Sağlam · Emrah Güler · Birol Akbaş · Üftade Güner

Received: 18 June 2023 / Accepted: 31 July 2023 / Published online: 17 August 2023
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Abstract This study was carried out to determine the effectiveness of modified thermotherapy and shoot tip culture in eliminating grapevines from grapevine fleck virus (GFkV), and grapevine leafroll closterovirus (GLRaV1, GLRaV2, and GLRaV3) all of which are required for certified sapling production. The infected grapevine scions were transferred into 10-liter plastic containers filled with a 1:1 mixture of perlite and peat (v/v), rooted, and saplings were grown. For 40 days, the grapevines were subjected to a modified thermotherapy treatment that included 8 h of darkness at 60–70% relative humidity and 32 °C temperature and 16 h of light (4000–5000 lx) at 60–70% relative humidity and 40 °C temperature. The materials were subjected to shoot tip culture procedures after thermotherapy. All materials were tested again at the end of the acclimatization period. The entire grapevines were virus-free at the end of the process. The survival rates of the grapevines varied. The 41B rootstock had a survival rate of 100%, and the rest had at least one surviving plant out of six. The proposed thermotherapy approach in this study has a high potential for sanitary grapevine sapling propagation when combined with rapid multiplication via *in-vitro*.

Keywords Grapevine · Virus · Thermotherapy · Shoot tip culture

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12600-023-01101-x.

H. Sağlam (✉)
Department of Horticulture, Faculty of Agriculture, Pamukkale University, Denizli, Türkiye
e-mail: hayris@pau.edu.tr

Ö. Çalkan Sağlam
Department of Horticulture, Faculty of Agriculture and Natural Science, University of Bilecik Şeyh Edabali, Bilecik, Türkiye

E. Güler
Department of Horticulture, Faculty of Agriculture, Bolu Abant Izzet Baysal University, Bolu, Türkiye

B. Akbaş
Plant Protection Central Research Institute, Ankara, Türkiye

Ü. Güner
Plant Protection Research Institute, İzmir, Bornova, Türkiye

Introduction

Türkiye stands as a prominent vine-growing country with a rich grapevine genetic potential thanks to its advantageous geographical position encompassing the Mediterranean and Near-East gene centers (Güler, 2023), including both wild (*Vitis vinifera* ssp. *sylvestris*) and cultivated (*Vitis vinifera* ssp. *sativa*) grapevines (Güler et al., 2023). Turkey is considered a gene center and the cultural origin of the renowned grapevine species, *Vitis vinifera* L., with a viticulture

history dating back approximately 8000 years (Dong et al., 2023). The productivity and quality of grapes are greatly influenced by cultural practices, pests, and diseases (Di Vittori et al., 2018). Pests and diseases can lead to significant losses in yield and quality despite appropriate cultural practices (Çelik, 2022). Viral diseases play a crucial role across the factors affecting vineyard productivity and quality, causing substantial losses in grapevines (Cabaleiro et al., 2023). Hence Türkiye is among the grapevine gene centers, viral infections can be widely observed in its diverse genetic pool.

Plant viruses can be detected in various plant tissues using a combination of serological and molecular methods (Çelik et al., 2023). Various virus species such as alfalfa mosaic virus (AMV), arabis mosaic virus (ArMV), grapevine fanleaf virus (GFLV), tomato black ring virus (TBRV), grapevine leaf roll-associated virus (GLRaV), grapevine fleck virus (GFkV), raspberry ringspot virus (RpRSV), GLRaV 1,2,3 and 4–9 have been identified using different methods in different regions of Türkiye (Özaslan et al., 1991; Akbaş & Erdiller, 1993; Özaslan & Yılmaz, 1994, 1995; Çaglayan, 1997; Akbaş & Erdiller, 1998; Kaya & Erilmez, 2014). In the vineyards of the Southeastern Anatolia region, GLRaV, particularly GLRaV1 and GLRaV3, were identified as the most prevalent viral diseases using serological methods (Özaslan et al., 1993). Viral infections by at least one of the GLRaV1,2,3,5,6, and 7 were detected in 92.7% of vineyards in the Thrace region using mechanical and serological techniques (Azeri & Çiçek, 1995; Koklu et al., 1998). In recent years, Karadeniz et al. (2018) noted infections by GLRaV-1 (9.7%), GFLV (0.3%), ArMV (5.7%), strawberry latent ring spoty virus (SLRSV) (4.7%) GFkV (0.7%), and grapevine virus A (GVA) (5%). Moreover, Türkmen and Ertunç (2019) reported a 100% viral infection rate by at least one of the GLRaV1, GLRaV2, GLRaV3, GLRaV4, GLRaV6, GLRaV7, and GLRaV9 in the Eastern Anatolia Region, followed by 69.74% in the Marmara Region, 75% in the Aegean Region, and 62% in the Central Anatolia Region.

Implementing phytosanitary measures that minimize the transmission of viruses through vectors and vegetative propagation is crucial (Topkaya et al., 2023). Grapevines are predominantly propagated vegetatively, which allows the widespread distribution and dissemination of virus species throughout the plant, leading to the complex development of diseases. Currently, more than 80 distinct viruses have been identified to infect grapevines (Fuchs, 2020), the leafroll group being one of the most significant groups (Maliogka et al., 2015). Controlling viruses through chemical treatments is impractical in plants. While controlling vectors through chemicals is theoretically possible, the diverse range of invertebrate organisms that act as vectors pose a considerable challenge to retaining measures (Tan et al., 2022).

All grapevines are presumed potentially infected with viruses in uncontrolled open conditions. Therefore, conducting virus testing in sapling production and eliminating infected materials is crucial to ensure successful production activities (Diaz-Lara et al., 2023). Various techniques are utilized for sanitating virus-infected materials (Adil et al., 2022). Thermotherapy is one of the widely used methods that involves growing and sanitating grapevines at temperatures that can kill or inactivate viruses. This technique focuses on inactivating the viruses rather than removing them (Wang et al., 2018). Researchers suggested thermotherapy, shoot tip culture, meristem culture, or a combination of these approaches to sanitate virus-infected plants (Skiada et al., 2009). Naik and Buckseth (2018) emphasized that the size of the meristem piece plays a crucial role in obtaining virus-free plants. Typically, researchers recommend using 0.5-1.0 mm meristem tissues with 2–3 prophylls for successful results (Azad et al., 2020). Additionally, reducing the size of the shoot tip was reported to increase obtaining healthy plant chance (Bettoni et al., 2021).

This study was carried out to assess the effectiveness of the combination of the shoot tip culture and modified thermotherapy method to eliminate common viruses in certain European grapevines and American rootstocks.

Material and method

Material

Twenty-one clones of six *V. vinifera* cultivars (Çal Karası, İpek, Osmanca, Pembe Gemre, Razakı, Yuvarlak Çekirdeksiz) and four clones of two

American rootstocks (41B and 420 A) that was selected by a clonal-selection study carried out in Turkey were used as research material (Table 1).

Methods

All materials were tested for GFLV, GFkV, and GLRaV1, GLRaV2, GLRaV3, RpRSV, and SLRSV, which are essential for certified sapling production in perennial plants. DAS-ELISA (double antibody sandwich – enzyme-linked immunosorbent assay) and RT-PCR (reverse transcription-polymerase chain reactions) were utilized in the testing and identification of the viruses.

DAS-ELISA assay

About 50–100 cm scions having at least 3–4 nodes were taken from shoots at different sides of the vines. Samples were wrapped with clean humid cloths, placed into plastic bags, and brought to the laboratory in an ice box. All study materials were tested for the two most harmful groups of grapevine viruses, grapevine leafroll-associated viruses 1, 2, 3 and grapevine fanleaf virus, by an ELISA (Clark & Adams, 1977) assessed on phloem tissues of annual shoots using commercial kits (Bioreba AG, Reinach, Switzerland) according to the Commission Directive 2005/43/EC amending the Annexes to Council Directive 68/193/EEC on the marketing of grapevine propagation material.

RNA isolation

RNA was isolated from the most concentrated virus samples from positive grapevines in ELISA tests according to Nassuth et al. (2000). Briefly, 0.1 g of the plant samples were taken and extracted in 5 ml of RNA extraction buffer containing 6 M guanidine thiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 1 M potassium acetate, 2.5% PVP-40, and 1% mercaptoethanol (Foissac et al., 2000). Then, the extract was centrifuged at 8000 rpm for 10 min and stored at -70 ℃ until use. For direct RT-PCR, purified extracts were diluted to 2.5-fold with sterile distilled water to a final volume containing 1% Triton™ X-100 (transcription) and incubated at 65 ℃ for 5 min. The viral RNAs separated from the virions were made ready for use in the reverse transcription reaction. The silica gel method was implemented as follows; 100 mg of plant tissue crushed with liquid nitrogen was mixed by adding 1 ml of extraction buffer. 500 µl of the mixture was taken and mixed with 100 µl of 10% sodium lauryl sarcosine solution. This mixture was mixed and incubated at 70 ℃ for 10 min and then kept on ice for 5 min. Then, it was centrifuged at 14,000 rpm for 10 min. After transferring 300 µl of supernatant to a new tube, 150 µl of ethanol, 25 µl of silicon dioxide, and 300 µl of 6 M sodium iodide were added. After incubation for 10 min with stirring at ambient temperature, the mixture was centrifuged at 6000 rpm for 1 min. The supernatant was then discarded, and the pellet was dissolved in 500 µl of fresh buffer (10 mM Tris-HCl containing 0.05 mM EDTA, 50 mM NaCl, and 50% ethanol). After repeating this process twice, the pellet was dissolved in 150 µl of RNasefree water. Then it was kept at 70 ℃ for 4 min and centrifuged at 14,000 rpm for 3 min. Finally, the supernatant was taken into a new tube and stored at -20 ℃.

Table 1 The clone numbers of grape cultivars and rootstocks used in the study

No	Variety/rootstock name	Clone number
1	Razakı	16, 18, 21
2	İpek	4, 13, 25
3	Osmanca	21, 38, 39, 40
4	Pembe Gemre	6, 11, 12
5	Çalkarası	1, 10, 12, 18, 29
6	Yuvarlak Çekirdeksiz	5, 7, 8
7	420 A (Rootstock)	13, 19
8	41B (Rootstock)	8, 13

RT-PCR amplification

The RNA concentration was determined for each sample, and the RNA quality was checked in the agarose gel. PCR amplification was performed as a one-step RT-PCR with a primer set characterized for virus RNA (Martin et al., 2005), which was specific to a region near the 3' end of the virus coat protein gene. The sequence and annealing temperature of the viruses used for this purpose were determined according to Rowhani et al. (1993) and Martin et al. (2005). The acquired product was run on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light. The primer pairs used in the study are given below (Table 2).

Table 2 The primers used in the study and their informations

Virus	Primer	Sequance (5'–3')	References
GLRaV-1	LR-1	F: CGACCCCTTTATTGTTTGAGTATG R: GTTACGGCCCTTTGTTTATTATGG	Osman et al. (2007)
GLRaV-2	LR-2	F: ATAATTCGGCCCTTTGTTTATTATGC R: GCCTCCGCGCAACTAATGACAG	Martin et al. (2005)
GLRaV-3	LC1	F: CGCTAGGGCTGTGGAAGTATT R: GTTGTCCCGGGTACCAGATAT	Jones et al. (2014)
GLRaV-7	C 995 H 587	AAGCCTGACCTAGCTATCTTGG GACAAATGGCACAGTACG	(www.plantpath.wisc.edu technıcal sheet)
GFLV	GFLV-V1	F: ACCGGATTGACGTGGGTGAT R: GGTTTCAACCAAAGGGTTCT	Osman et al. (2008)
GFkV	Fk19 Fk3	GTCCTCCTACACCTCCCTGTCCAT' CCTCATCCGCGGAGTTATCGAAT	Sabanadzovic et al. (2001)
RpRSV	RSP 48	F: AGCTGGGATTATAAGGGAGGT R: CCAGCCGTTCCACCACTAAT	Martin et al. (2005)
SLRSV		F: CCTCTCCAACCTGCTAGACT' R: AAGCGCATGAAGGTGTAACT	Martin et al. (2005)

GLRaV; Grapevine leafroll-associated virus, GFLV: Grapevine fanleaf virus, GFKV: Grapevine fleck virus, RpRSV: Raspberry ringspot nepovirus, SLRSV: Strawberry latent ringspot virus, F: forward primer, R: reverse primer

Sanitation studies

The materials identified as infected at the end of testing procedures were subjected to sanitation. A modified thermotherapy method was utilized in the sanitation process. Here, infected materials were initially subjected to thermotherapy treatments. Scions were planted in 1:1 perlite-peat (v/v) fulfilled 10-liter plastic pots for rooting and shoot development. The materials planted in pots developed a short shoot and were placed into thermotherapy cabins. Samples were subjected to 8 h of dark treatments at 60–70% relative humidity and 32 ℃ temperature and 16 h of light treatments (4000–5000 lx) at 60–70% relative humidity and 40 ℃ temperature for 40 days.

Newly developed shoot tips were taken and shortened to 1.5–2.0 cm. Shoot tips were then sterilized by immersing them into 0.6% Na-hypochlorite solution for 20 min and rinsing them thrice with sterile distilled water. The sterile cabin was also sterilized with an ultraviolet lamp. All the tools used in the study (forceps, lancets, etc.) were sterilized in an oven at 180 ℃ for 30 min. The Murashige and Skoog (MS) nutrient medium was sterilized the same as materials and tools by autoclaving at 1 atm pressure and 121 ℃ temperature for 30 min in tubes. Surface-sterilized shoot tips were taken as 0.5-1 cm in size and planted in jars filled with sterilized MS. Jars were placed in a climate chamber at 25–27 ℃ temperature, 60–70% relative humidity, and 2000–3000 lx lighting for development.

The plants developed in jars for a month were transferred to benzyl amino purine (BAP)-supplemented half-strength MS medium. Shoot and root development was promoted through transplanting each month. Rooted plants were adapted to outdoor conditions with misting treatments (Thomas & Schiefelbein, 2001), and then they were transplanted into a 1:1 soil-perlite mixture (v/v) in screen houses. After thermotherapy, meristem culture, and acclimatization, the plants were again subjected to DAS-ELISA and RT-PCR when they reached seven real leaves.

Statistical evavluations

The data belonging to shooted explants, shoot numbers, and root numbers were subjected to hierarchical clustering using Ward's method by the "ggplot2" package of R software (Wickham, 2011).

Results

After the initial tests, most materials were infected with GLRaV1, GLRaV2, GLRaV3, and GFkV viruses, while no infection of GFLV, RpRSV, or SLRSV was detected. Twelve grapevines were infected with GFkV out of 19 clones, while six of them were also infected with GLRaV1. Four

Fig. 1 The initial testing results and the infection incidences after sanitation treatments against viruses. IBT: Infection status before treatment, IAT: Infection status after treatment,. Clones having more than one color were infected by multiple viruses. Red, blue, green, and purple indicate infections GLRaV-1, GLRaV-2, GLRaV-3, and GFkV, respectively, while grey represents non-infected plants

No	Variety/rootstock	Clone	IBT				IAT
1	Razakı	16					
2	Razakı	18					
3	Razakı	21					
4	İpek	4					
5	İpek	13					
6	İpek	25					
7	Osmanca	21					
8	Osmanca	38					
9	Osmanca	39					
10	Osmanca	40					
11	Pembe Gemre	6					
12	Pembe Gemre	11					
13	Pembe Gemre	12					
14	Çalkarası	1					
15	Çalkarası	10					
16	Çalkarası	12					
17	Çalkarası	18					
18	Çalkarası	29					
19	Yuvarlak Çekirdeksiz	5					
20	Yuvarlak Çekirdeksiz	7					
21	Yuvarlak Çekirdeksiz	8					
22	420A	13					
23	420A	19					
24	41B	8					
25	41B	13					

grapevines were infected with GLRaV2, and 14 were infected with GLRaV3 (Fig. 1).

Four clones from the varieties (Çalkarası-29, İpek-25, Osmanca-38, Razakı-16) and two of the 420 A rootstock were free from any tested viruses, while six clones of varieties (Çalkarası-1, Osmanca-21, Razakı-18 and 21, Yuvarlak Çekirdeksiz-20 and 21) and both clones of the 41B rootstock were infected with a single virus. The clones Razakı 18 and 21, Osmanca 21, Çalkarası 1, and Yuvarlak Çekirdeksiz 7 and 8 were only infected with GLRaV3, while the 41B clones (clones 8 and 13) were infected with GFkV. Two clones of the Pembe Gemre variety (clones 6 and 11) were infected with four viruses, while the other clone (clone 12) was infected with three viruses except for GLRaV2 (Fig. 1).

Subsequently, all the materials identified as infected underwent virus elimination procedures, including thermotherapy treatment, followed by shoot-tip cultures. Around 10–12 weeks after treatments, when they reached seven leaves, all materials were subjected to further testing for viruses with DAS-ELISA and RT-PCR, and all materials were confirmed to be virus-free or "clean" (Fig. 1).

At least one plant survived in each clone after the shoot-tip culture. However, there were variations in the viability and survival rates among different clones. Only one explant perpetuated its viability in Razakı and İpek cultivars from *V. vinifera* species and the 420 A clones from American rootstocks. Clones 6 and 11 of the Osmanca variety and 38 and 40 of the Pembe Gemre variety had relatively lower numbers of viable plants after the shoot tip culture. On the other hand, four clones of the Çalkarası variety exhibited high survival rates, while Çalkarası-18 had only one living explant. The 41B American rootstock clones achieved a 100% survival rate and yielded high shoot and root numbers. 41B rootstock's clone 8 had the highest total root number (26 roots), while Clone 1 of the Çalkarası displayed the highest total shoot number (60 shoots). Pembe Gemre's clone 12 also yielded remarkable results with 21 shoots and roots (Supp. Table 1).

The varieties and rootstocks were categorized into two main clusters each containing two subclusters based on shooted explant count, shoot and root

numbers. The first cluster (A) consisted of 22 grapevines, while the other three were in the second cluster (B). The first subcluster of A (A1) mainly comprised the clones of varieties and rootstocks with the lowest survival rates, and the second subcluster (A2) exhibited variations in terms of the numbers of survived explants, shoots, and roots. The second main cluster (B) demonstrated distinct differences in shoot and root numbers across the varieties. The Çalkarası clone-1 formed the second subcluster of B on its own, while the Pembe Gemre 12 and 41B 8 were included in the first subcluster (B1) (Fig. 2).

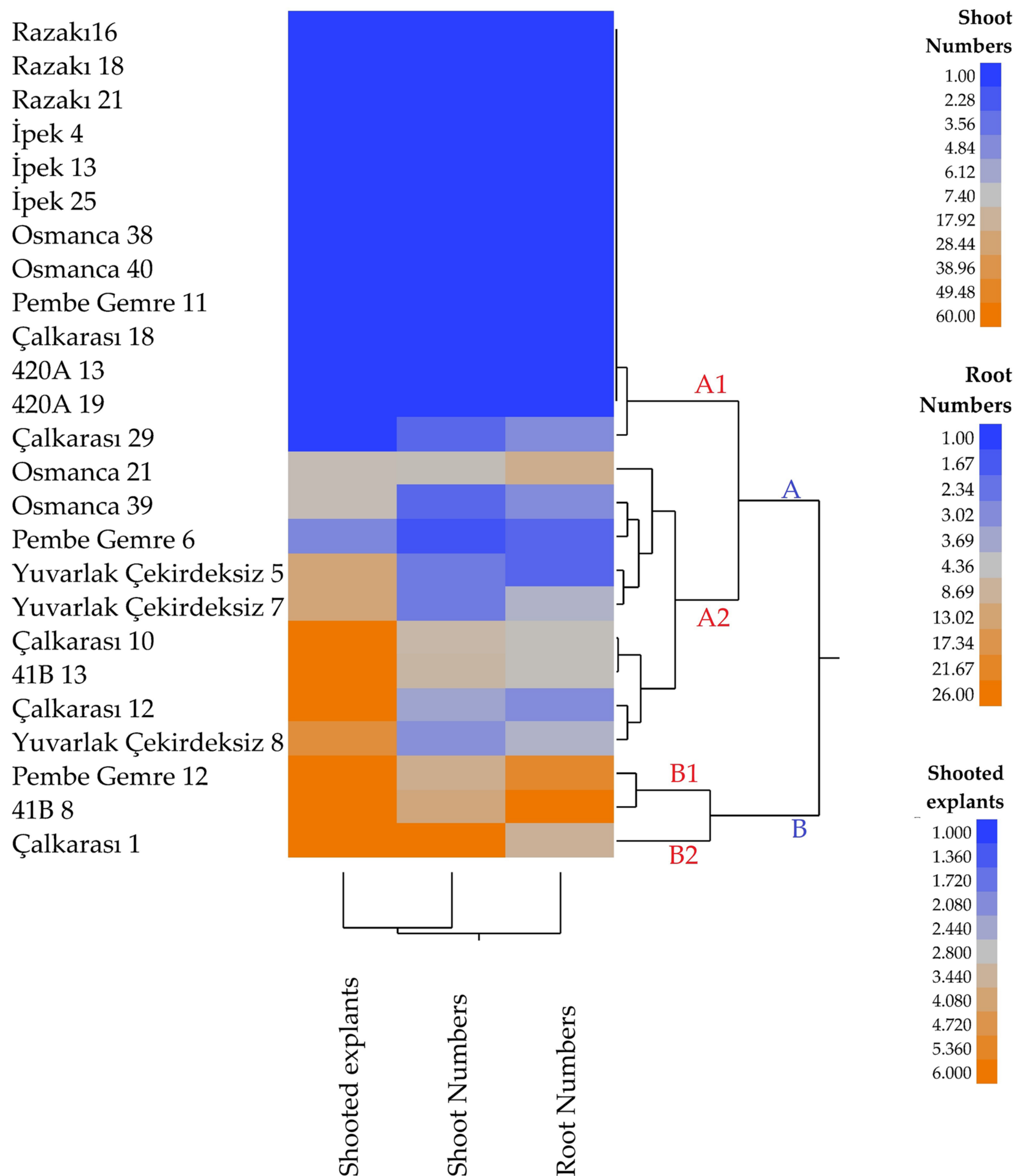

Fig. 2 The clustering of clones is based on the numbers of explants with shoots and shoot and root numbers after the shoot tip culture

Discussion

Vegetative techniques are commonly used for production in all deciduous species, emphasizing the importance of using virus-free stocks to prevent the transmission of pathogens to new generations (Rowhani et al., 2005). In Turkey, the presence of GLRaV1 and GLRaV3 in the same grape varieties was identified through ELISA (Gürsoy, 1991).

Heat treatment has been shown to inhibit viral RNA synthesis and reduce the migration of virus particles into the apical meristem of plants (Hu et al., 2015). Rising temperatures, such as 38 ℃, can lead to the degradation of viruses in shoot tips and leaves, possibly due to enhanced RNA silencing mechanisms (Wang et al., 2008). This phenomenon becomes more significant at higher temperatures (Chellappan et al., 2005). Previous studies have utilized different temperature and time combinations for eliminating grapevine viruses. For instance, Leonhardt et al. (1998) applied 35 ℃ for 57 days to 5 mm shoot tips infected with GLRaV, GFLV, and AMV, repeating the treatments twice, and samples were found to be negative in DAS-ELISA tests. However, in some studies, complete virus sanitation was not achieved (Maliogka et al., 2009; Panattoni & Triolo, 2010; Wang et al., 2018). While grapevine viruses can be inactivated through thermotherapy at 38 ℃, the suppression of root and shoot development can lead to chlorosis and other deformations of leaves (Miljanić et al., 2022). Therefore, combining meristem culture with thermotherapy has been suggested to achieve virus elimination in cases where thermotherapy alone is insufficient.

Thermotherapy studies in garlic at 37 ℃ for 35 days resulted in approximately 70% clean material in immunosorbent electron microscopy tests, and about 90% of the material was clean when the temperature was raised to 40 ℃ (Torres et al., 2000). Thermotherapy treatments at 37 ± 2 ℃ for 40 days (Mahmoud et al., 2009), or 16 h of light at 42 ℃ and 8 h of dark at 39 ℃ for four weeks, successfully eliminated viruses in potatoes (El Far & Ashoub, 2009). Hot water treatments at 50 ℃ and 55 ℃ for 0.5, 1, and 2 h resulted in approximately 25% of the samples still testing positive at 55 ℃ for 0.5 h in tomatoes infected with the pepino mosaic virus (PepMV), while 1 and 2 h of treatment completely inactivated PepMV (Ling, 2010). The findings of earlier studies align with the clean material obtained in the present study. The main difference between this and previous studies is that in the current study, we achieved virus-free material through shoot tip culture, whereas meristem culture was typically employed after thermotherapy in previous studies. The shoot tip culture employs the utilization of cultivating shoot tips (2 cm or less), and it is considerably easier to utilize than the meristem culture since only the apical dome and a few leaf primordia are separated and placed in nutritional media during meristem culture (Ferdous et al., 2015). Also, this study's temperature regime in thermotherapy treatments (16 h of light at 40 ℃ and 8 h of dark at 32 ℃) differed from earlier studies. This study successfully recovered grapevines from GLRaV1, GLRaV2, GLRaV3, and GFkV using thermotherapy and shoot-tip culture methods.

Conclusion

We presented an alternative and effective method for virus elimination in both European and American grapevines. We used a thermotherapy treatment of 8 h of dark treatments at 60–70% relative humidity and 32 ℃ temperature and 16 h of light treatments (4000–5000 lx) at 60–70% relative humidity and 40 ℃ temperature for 40 days, and 1.5–2.0 cm shoot tips for in vitro propagation. All grapevines studied were successfully sanitated. Since the meristem culture, the commonly utilized method in sanitation studies, is more complicated than shoot-tip culture and requires expertise and experience, the results are crucial for virus-free sapling production in grapevine. Using the shoot tip culture method instead of the tedious meristem culture will ease the feasibility of such studies.

Author contributions H.S., Ö.Ç.S, B.A., and Ü.G. carried out the project, H.S., Ö.Ç.S, B.A., Ü.G., and E.G. wrote the main manuscript text, E.G. prepared the analyzed the data and figures, all authors reviewed the manuscript and agreed the final verison.

Funding This study was supported by funds obtained from the project "TUBITAK 107G116".

Data availability The corresponding author will supply the data upon reasonable requests.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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