Electrotherapy, thermotherapy, chemotherapy, and cryotherapy to regenerate Prunus armeniaca L. free of ACLSV, ApMV, and TRSV

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Abstract

In the present study, *in vitro* regenerated shoot tips of three apricot cultivars namely Qaysi , Shams and Ordubad , already infected with *Apple chlorotic leaf spot Trichovirus* (ACLSV), *Apple mosaic Ilarvirus* (ApMV), and *Tobacco ring spot Nepovirus* (TRSV), were exposed to electrotherapy (0, 30, 40, 50, and 100 mA), thermotherapy (38°C for 7 days), chemotherapy (ribavirin at 0, 25, 50, 75, and 100 mg L^{-1}), or droplet-vitrification cryotherapy (40 min at 0°C prior to -20°C and − 80°C for 10 and 15 min, respectively) to achieve virus-free plants. Although electrotherapy with current intensities more than 40 mA led to drastic decrease in explants' viability, a 40–60% virus removal rate was noticed depending on the type of virus and the variety tested. Amongst various shoot tip sizes exicised, 1.0–2.0 mm explants exhibited by far more survival (60–80%) and virus eradication rate (90–100%) following thermotherapy. The explants' survival and proliferation rates also decreased with increment of ribivirin concentration in the culture medium as compared to the untreated cultures. The rate of virus elimination, however, inclined by 75–100% upon exposure to 25 mg L^{-1} ribavirin. According to our results, production of virus-free regenerants would be feasible in *Prunus armeniaca* L. providing proper therapeutic methods are adopted as regards the type of infecting virus and the host variety.

Introduction

Apricot (*P. armeniaca* L.), belonging to the *Prunus* genus of the *Rosaceae* family, now has been cultivated worldwide due to its early harvesting season, unique aroma, pleasant taste, high nutritional value, and other multiple uses (Jiang et al. 2019).

Plant diseases have long been regarded as the major constraint for economic production worldwide. Stone fruits are natural hosts for several detrimental graft-transmissible agents including viruses, viroids, phytoplasmas, and some systemic bacterial and fungal pathogens (Hauptmanová and Polák 2011; Koubouris et al. 2007). Amongst which, viral diseases can cause enormous economic losses, reportedly exceeding 30 billion dollars per annum (Hilaire et al. 2022). Considering the fact that no definitive method for virus eradication exists under the field conditions (Wang et al. 2018; Thresh 2006), prevention of virus introduction through cultivating virus-free material would be of paramount economic importance in the horticulture industry.

Various *in vitro* eradication and therapy procedures including meristem tip culture, thermotherapy, chemotherapy, electrotherapy, cryotherapy, alone or in combination, have been proposed to roll out virus-free plants in different fruit tree species (Mathew et al. 2021; Wang et al. 2018; Kazemi et al. 2020; Kaya 2021). Being undifferentiated, genetically stable, and having tremendous growth potential, meristem tips offer an ideal explant to regenerate plants free of viruses from infected propagules (Retheesh and Bhat 2010; Wang et al. 2016; Wu et al. 2020). This technique, however, is time consuming, labor-intensive, and incapable of eliminating some viral diseases (Meybodi et al. 2011).

Compared to meristem-tip culture, electrotherapy is a relatively less demanding and more economical for virus elimination (Maliogka et al. 2015dărău et al. 2014). This approach has been successfully employed to

eliminate viral diseases in both herbaceous and woody plants (Singh and Kaur 2016; Kaur et al. 2019; Nerway et al 2020). Depending on the voltage applied, duration of exposure, and plant explant utilized, electrotherapy has exhibited viral elimination efficiency of more than 50% (reviewed by Adil et al. 2022). Although its underpinning mechanism is still obscure, it has been postulated that electrotherapy decreases internal viral contamination through degrading their nucleoprotein plausibly *via* applying a continuous electric current to the exposed plant tissues (Sastry and Zitter. 2014).

Shoot tip cryotherapy has also been scientifically proven to be an efficient and reliable strategy for eliminating viruses from infected plant materials (Wang et al. 2017; Bettoni et al. 2019; Zhao et al. 2019; Farhadi-Tooli et al. 2021; Kaya 2021; Mathew et al. 2021). In this technique, the excised explants are briefly exposed to the liquid nitrogen (at − 196°C) which ultimately leads to removal of infected differentiated cells by the ultra-low temperature, leaving cytoplasmic meristematic cells to regenerate virus-free plantlets (Wang and Valkonen 2009).

Exposing the explants to heat shock (thermotherapy) is also another approach to decrease virus particle movements towards the apical meristem cells and inhibit viral replication (Hu et al. 2015). Concrete evidence have shed light on the fact that viruses can be effectively eliminated after exposing explants to heat treatment (30–42°C) for two to eight weeks in different plant species (Şeker et al 2015; Wang et al. 2018; Zarghami and Ahmadi 2022). However, host plants are often vulnerable to the high temperatures, and prolonged durations can reduce the host viability drastically (Hu et al. 2015). Therefore, the optimal temperature regimes should allow the treated plant to survive on the one hand, yet inactivate the present viruses, on the other (Wang et al. 2018).

Chemotherapy, treating infected plants using chemicals, has also been successfully employed to eliminate viral disease in wide range of plant species. Amongst different chemicals utilized, ribavirin (1-β-ribofuranosyl-1,2,4,-triazole-3- carboxamide, trade name: virazole) is the most frequently used agent in stone fruits (Howell et al. 2001; Paunovic et al. 2007). It is a well - known member of the nucleoside anti-metabolite drugs hindering duplication of viral hereditary material and protein synthesis (Meier et al. 2003; Leyssen et al. 2005). Not to mention, novel promising chemical compounds such as sodium nitroprusside (SNP) have also been applied in pome fruits for virus eradication (Kazemi et al. 2020).

Selection of suitable sanitation method depends generally on plant species and the structural and biological characteristics of the targeted virus (Ben Mahmoud et al. 2017). In this study, shoot tips of *P. armeniaca* L. were either exposed to electrotherapy (0, 30, 40, 50, and 100 mA), thermotherapy (38°C for 7 days), chemotherapy (ribavirin at 0, 25, 50, 75, and 100 mg L^{-1}), or droplet-vitrification cryotherapy (40 min at 0°C prior to − 20°C and − 80°C for 10 and 15 min, respectively) in order to determine the most efficient *in vitro* procedure(s) for production regenerants free of ACLSV, ApMV, and TRSV.

Materials And Methods

Plant materials

Three apricot (*P. armeniaca* L.) vars. namely Qaysi , Shams , and Ordubad , kindly provided by Horticultural Science Research Institute (HSRI), were aimed to establish virus eradication procedures.

Initial Virus Infection Assessment

Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA)

Leaf extract from the donor trees were initially tested for viral infection by DAS-ELISA using virus-specific antibodies (Bio Reba, Switzerland) according to manufacturer's instruction. About 200 mg of leaf samples were finely ground in 4 ml of extraction buffer using a pestle and mortar. The mixtures were then centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was discarded and the upper phase was kept at −20°C until needed. Then, 200 µl of immunoglobulin G antibody along with coating buffer were added to each well of the ELISA plate and maintained at 4°C overnight. The next day, 200 µl of leaf extract was added to each well and again kept at 4°C overnight. After washing with washing buffer, conjugate buffer and specific enzyme of each virus were added to each well and the whole plate was incubated at 30°C for 5 h. Finally, 200 µl of substrate (suitable for coloring) was added to each well, maintained in the dark at room temperature for 1–2 h, and the plate was read using an ELISA reader at 405 nm wavelength. Samples were considered positive only if their optical densities were twofold higher than the negative (virus-free) samples.

Reverse Transcription Polymerase Chain Reaction (Rt-pcr)

Reverse Transcription Polymerase Chain Reaction (RT-PCR)
The sanitary status of the donor trees were also confirmed using RT-PCR test. Total RNA from 200 mg of fine powdered young leaves and petioles was extracted (Zhang et al. 2013). Samples were initially washed with 1 mL washing buffer (8 mL 100% ethanol, 2 mL diethyl pyrocarbonate (DEPC)-treated water and 200 µL β-mercaptoethanol) and centrifuged at 8000 rpm for 10 min at 15°C. The supernatant was decanted and 1 mL extraction buffer (1 ml Tris HCl (1M), 0.2 g polyvinyl pyrrolidone (PVP), 0.86 g NaCl, and 400 µL EDTA (0.5 M)) was added to the samples and vortexed. Then, 7 µL proteinase K and 12 µL β-mercaptoethanol were added to the samples. Subsequently, the samples were incubated at 65°C for 30 min and centrifuged at 8000 rpm for 15 min. In the next step, the equal volume of chloroform: isoamyl alcohol (IAA) (24:1, v:v) was added to the supernatant and centrifuged at 12000 rpm for 15 min at 4°C. Supernatant was mixed with lithium chloride 10 M (v/v, 1:3) and incubated at -20°C overnight. Samples were centrifuged at 12000 rpm for 30 min at 4°C and the pellets were washed twice with lithium chloride (4 M) followed by re-suspending in 300µL Tris HCl (10 mM and pH 7.5) containing potassium acetate (3 M) for 30 min at -20°C. Thereafter, tubes were centrifuged at 12000 rpm for 15 min, washed with 1 mL 100% ethanol (-20°C), and centrifuged again for 20 min. The pellets were finally washed with 75% ethanol, air dried, and dissolved in 30 µL DEPC-treated water.

First strand cDNA was synthesized from 1 µg of total extracted RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA, K1622) according to the manufacturer's instruction. The cDNAs were

checked for partial amplification of *Rubisco* (ribulose bisphosphate carboxylase chloroplast ribosomal), as a housekeeping gene, prior to virus diagnosis using specific primer pairs (Table 1). The PCR amplifications were carried out in a 96-well thermal cycler (Applied Biosystem, USA) using the following cycles: an initial denaturation of cDNAs at 95°C for 15 min, 35 cycles of amplification (denaturation at 94°C for 30s, target-specific annealing temperature (Table 1) for 45s, extension at 72°C for 1 min), and the final extension step at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel in TAE buffer (1X), stained with 0.5 µL mL^{-1} ethidium bromide, and visualized under 300 nm UV light (Gelduc., UK). The sizes of the amplified fragments were determined using Gen Ruler 1 kb plus DNA Ladder (Thermo Scientific, USA).

Table 1
Primers used for partial amplification of viruses and housekeeping gene using RT-PCR

Primer name	Sequence (5'→3')	Amplicon (bp)	Annealing temp. (°C)	Target gene	References
ArMV-F1	TTGGTTAGTGAATGGAACGG	427	60	*CP*	Grieco et al. 2000
ArMV-R1	CAAGCTATCATGTGGGCAAA				Naderpour and Shahbazi 2014
ApMV-F1	CGTAGAGGAGGACAGCTTGG	450	55	*CP*	Hasan et al. 2006
ApMV-R1	CCGGTGGTAACTCACTCGTT				
ACLSV-F2	TTCATGGAAAGACAGGGGCAA	677	55	*CP*	Menzel et al. 2002
ACLSV-R2	AAGTCTACAGGCTATTTATTATAAGTCTAA				
TRSV-F2	CAGGGGCGTGAGTGGGGGCTC	580	58	*RdRp*	Fuchs et al. 2010
TRSV-R2	CAATACGGTAAGTGCACACCCCG				
ToRSV-F2	ACTTCTGAAGGCTACCCGTT	320	58	*RdRp*	Fuchs et al. 2010
ToRSV-R2	CCACCACACTCCACCTACC				
PNRSV-F3	GCCGAATTTGCAATCATACCC	599	54	*MP*	Naderpour and Shahbazi 2014
PNRSV-R3	ACTTCGGTCTTGAATTCGAT				
PPV-F1	GGAATGTGGGTGATGATGG	345	60	*Nib*	Jarošová and Kundu 2010
PPV-R1	CTCTTCTTGTGTTCCGACGTTTC				
PDV-F2	CAACGTAGGAAGTTCACAG	517	50	*RNA3*	Sanchez-Navarro et al. 2005
PDV-R2	GCATCCCTTAAAGGGGCATC				

CP: Coat protein; RdRp: RNA dependent RNA polymerase; MP: Movement protein; Nib: Nuclear inclusion protein b; *Rbc1*: ribulose bisphosphate carboxylase chloroplast ribosomal.

Primer name	Sequence (5'→3')	Amplicon (bp)	Annealing temp. (°C)	Target gene	References
Rbc1–F	TACTTGAACGCTACTGCAG	186		*Rbc1*	Sanchez-Navarro et al. 2005
Rbc1–R	CTGCATGCATTGCACGGTG				

CP: Coat protein; RdRp: RNA dependent RNA polymerase; MP: Movement protein; Nib: Nuclear inclusion protein b; *Rbc1*: ribulose bisphosphate carboxylase chloroplast ribosomal.

In vitro **shoot stock culture**

Shoots, measuring 1.5–2.0 cm in length and containing terminal and lateral buds from infected samples, were surface-sterilized with 70% ethanol (1 min), 2.5% nanosilver solution (10 min), and then with 1.2% sodium hypochlorite containing 1% Tween-20™ (v/v) (10 min), respectively. They were then rinsed three times in sterile distilled water each for five minutes to remove the traces of chemicals. Thereafter, shoots were transferred to the jars containing modified QL medium (Zare et al. 2020) supplemented with 2.22 µmol L^{-1} BAP, 2.88 µmol L^{-1} GA_3, 228.72 µmol L^{-1} Fe-EDDHA, 30.0 g L^{-1} sucrose, and 6.0 g L^{-1} agar. The pH of culture medium was adjusted to 5.8 ± 0.2 using NaOH (1N) or HCl (1N) prior to adding agar. Cultures were maintained at 24 ± 1°C under a 16-h photoperiod and daylight intensity of 3000 lux. Subcultures were performed once every four weeks and 14-subculture-old stock cultures were used as a source of plant material for virus eradication therapy experiments.

Virus Eradication Procedures

Electrotherapy

The *in vivo* shoots containing apical and axillary buds were harvested from the vars. Qaysi , Shams , and Ordubad and cut into 2.0–3.0 cm segments containing a single node (Fig. 1A). They were then immersed in NaCl (1 M) in vertical electrophoresis tank (WAVESYS VS20, UK) and exposed to electric current intensity of 0, 30, 40, 50 or 100 mA with 100 V for 15 min using power supply Enduro 300V (Labnet, USA). Thereafter, electro-treated shoot explants were surface sterilized and cultured on the modified QL medium (Zare et al. 2020) as described above. Finally, the *in vitro* shoot tips (0.2–0.7, 0.7-1.0, 1.0–2.0 mm) were excised using dissecting microscope SZM-2 (Siemens, DEU) under aseptic conditions and cultured on optimized QL medium for further growth and development.

Droplet-vitrification Cryotherapy

The *in vitro* regenerated apical shoot tips (0.2–0.7, 0.7-1.0, 1.0–2.0 mm) containing leaf primordia were excised from the 14-subculture-old stock cultures of vars. Qaysi , Shams , and Ordubad and subjected to the droplet-vitrification cryotherapy procedure. The excised shoot tips were cultured on the modified QL

medium for 24 h in the dark at 24 ± 2°C. Then, they were incubated on the liquid pre-culture medium [QL + 1 mg L^{-1} BAP + dimethyl sulfoxide (DMSO) 5%, proline 2%] for 24 h in the dark at 24 ± 2°C, and then transferred into 2-mL cryovials. The pre-cultured shoot tips were incubated in plant vitrification solution-2 (PVS2) [QL, 30% (v/v) glycerol, 15% (v/v) ethylene glycol, 15% (v/v) DMSO, 0.4 M sucrose, and pH 5.8] on the ice (0°C) for 40 min. Cryovials were incubated at − 20°C and − 80°C for 10 and 15 min, respectively. After exposure to liquid nitrogen for 1 h, the cryo-treated shoot tips were instantaneously warmed up by placing into water bath (40°C) for 1 min. They were then transferred into washing solution (1.2 M sucrose) for 30 min.

The treated and control explants (untreated apical shoot tips) were cultured on the modified QL medium at 24 ± 1°C in the dark for one week and finally transferred to the jars containing fresh modified QL medium (Fig. 1B).

Thermotherapy

The *in vitro* shoots (measuring 4.0–5.0 cm in length) of apricot cultivars Qaysi , Shams and Ordubad containing apical and axillary buds were excised, surface-sterilized, placed on the QL medium, and transferred to the growth chamber (SG600, Noor Sanat Ferdos, Iran) with 40% relative humidity and light intensity of 3000 lux. Cultures were exposed to 24 ± 1°C in the first day and temperature increased 2˚C within a week reaching 38 ± 1°C. The samples were kept for one week at this temperature, 16-h photoperiod, and light intensity of 3000 lux provided by cool white fluorescent lights. Apical meristems (0.2–0.7, 0.7-1.0, 1.0–2.0 mm) containing peripheral tissues and leaf primordia were excised from heat-treated shoots and cultured on the same medium. Cultures were maintained in a phytotron at the temperature of 24 ± 1ºC, light intensity of 3000 lux provided by high-pressure metal halide lamps, and 16-h photoperiod for further growth and development (Fig. 1C).

Chemotherapy

The 14-month old *in vitro* shoots were cultured on the QL supplemented with various doses of ribavirin (0, 25, 50, 75, or 100 mg L^{-1}). After four weeks, the survived meristems were excised and sub-cultured on the same ribavirin treatment and maintained in a phytotron at the temperature of 24 ± 1ºC, light intensity of 3000 lux provided by high-pressure metal halide lamps, and 16-h photoperiod. After four weeks, the survived explants were transplanted on the ribavirin-free medium for proliferation. Then, the *in vitro* shoot tips (0.2–0.7, 0.7-1.0, 1.0–2.0 mm) were excised using dissecting microscope SZM-2 (Siemens, DEU) under aseptic conditions and cultured on optimized QL medium for further growth and development.

Acclimatization

Culture vessels containing plantlets with well-developed shoots and roots (Fig. 1D) were initially incubated in a bath water (30°C) for 10 min to reduce the solidity of agar gel. Then, the plantlets were taken out from the vessels and the remaining traces of agar were removed by washing the roots with distilled water. The

roots of plantlets were trimmed off and transferred to 200-mL plastic pots containing autoclaved perlite: peat moss (1:1; w:w), and maintained at greenhouse condition with temperature of 24 ± 1°C and 90% relative humidity. So as to prevent evaporation, a transparent disposable plastic cup was placed on each plantlet. After one week, holes were made in cups, and after two weeks, they were entirely lifted (Fig. 1E). 50 days later, the adapted plantlets were transferred to the pots containing soil: perlite (1:1; w:w) under the greenhouse condition.

Final Virus Infection Assessment

The DAS-ELISA and RT-PCR tests were performed for detection of ACLSV, ApMV, and TRSV in the regenerated plantlets according to manufacturer's instruction as described above.

Experimental Design And Statistical Analysis

All experiments were conducted in completely randomized designs (CRDs) with five replications (Petri dishes). Analysis of variance (ANOVA) was performed according to the general linear model (GLM) procedure using SAS statistical software (Institute Inc., Cary, NC, USA), and the means were compared using Duncan's Multiple Range Test (DMRT) at the $P \leq 0.01$ level of probability.

Results

Virus diagnosis

Samples collected from the vars. Qaysi , Shams , and Ordubad were positive for ACLSV, ApMV and TRSV according to both ELISA and RT-PCR assays. Agarose gel electrophoresis of PCR products indicated the amplification of monomorphic fragments of 450, 677, and 580 nucleotides belonging to partial genomic sequences of ApMV, ACLSV and TRSV, respectively. All three amplicons were also sequenced in two directions to confirm the belonging of amplified fragments to the assigned viruses. The sequences were submitted to NCBI under the accession numbers OP811253 (ApMV), OP811254 (TRSV) and OP811255 (ACLSV).

Effect Of Electrotherapy On Survival Rate Of Meristems

Our results indicated that the survival rate of explants relied significantly ($P \leq 0.01$) on the variety and voltages applied (Fig. 2). The highest percentage of active buds were observed in the control, irrespective of varieties tested. Although 40 mA voltage slightly increased survival rate in var. Qaysi when compared to control, the difference was not significant. Voltages higher than 30 mA proved to be so detrimental in var. Shams that no survival rate was observed in the voltages 40–100 mA.

Effect Of Cryotherapy On Survival Rate Of Meristems

According to our results, shoot tips smaller than 1.0 mm were not able to tolerate liquid nitrogen treatment, while 1.0–2.0 mm explants exhibited 10% viability in vars. Shams and Qaysi and 5% in var. Ordubad (Table 2).

Table 2
Effect of droplet-vitrification cryotherapy regimes on the survival rate (%) of virus-infected *in vitro*-cultured shoots of three varieties of *P. armeniaca* L.

Variety	Meristem size (mm)		
	0.2–0.7	0.7-1.0	1.0–2.0
Ordubad	0.0	0.0	5.0
Shams	0.0	0.0	10.0
Qaysi	0.0	0.0	10.0

Effect Of Thermotherapy On Survival Rate And Proliferation Of Meristems

Our results revealed that the survival rate increased drastically with increasing size of meristems in all three vars. tested, and the highest rates were achieved using 1.0–2.0 mm meristems while the lowest rates were observed in 0.2–0.7 mm meristems (Fig. 3).

A variety-dependent meristem proliferation rate was also observed in this study so that the highest number of auxiliary shootlets (per explant) were acquired in vars. Qaysi (3.27), Shams (2.56), and Ordubad (2.04), respectively (Table 3).

Table 3
Effect of thermotherapy regimes on the proliferation rate (number of regenerated shoots per explant) of virus-infected *in vitro*-cultured shoots in three vars. of *P. armeniaca* L.

Cultivar	Proliferation rate
Ordubad	$2.04 \pm 0.22^{b*}$
Shams	2.56 ± 0.46^{ab}
Qaysi	3.27 ± 0.33^{a}

* Means (± SE) followed by the same letters are not significantly different according to DMRT at $P \leq 0.01$.

Effect Of Chemotherapy On Survival Rate And Proliferation Of Meristems

The survival rate of explants declined with increasing concentration of ribavirin (Table 4). While almost 100% of explant were able to re-grow in the absence of ribavirin (control, 0 mg L^{-1}), no shootlets were attained in the presence of 100 mg L^{-1} in all varieties tested. Additionally, results of ANOVA indicated that inclusion of ribavirin to the culture medium exerted noxious impact on the proliferation rate i.e., the highest and lowest rate of proliferation were observed in the control (0 mg L^{-1}) and 100 mg L^{-1}, respectively (Table 4).

Table 4
Effect of chemotherapy regime (different ribavirin concentrations) on the survival rate (%) and proliferation (number of regenerated shoots per explant) in three vars. of *P. armeniaca* L.

Concentration (mg L^{-1})		Variety		
		Ordubad	Shams	Qaysi
Survival rate (%)	0	100 ^{a*}	100 ^{a}	100 ^{a}
	25	73.33 ^{b}	80.00 ^{b}	93.33 ^{b}
	50	40.00 ^{c}	46.66 ^{c}	60.0^{0} ^{c}
	75	13.33 ^{d}	13.33 ^{d}	20.0^{0} ^{d}
	100	0.00 ^{e}	0.00 ^{e}	0.000 ^{e}
Proliferation	0	2.33 ± 0.12 ^{a**}	2.33 ± 0.12 ^{ab}	4.08 ± 0.15 ^{a}
	25	1.25 ± 0.26 ^{b}	1.24 ± 0.25 ^{b}	2.75 ± 0.22 ^{b}
	50	0.66 ± 0.31 ^{bc}	0.50 ± 0.38 ^{bc}	0.75 ± 0.37 ^{c}
	75	0.08 ± 0.06 ^{c}	0.08 ± 0.06 ^{c}	0.17 ± 0.07 ^{c}
	100	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}

* Within a column, means followed by the same letters are not significantly different according to DMRT at $P \leq 0.01$.

** Within a column, means (± SE) followed by the same letters are not significantly different according to DMRT at $P \leq 0.01$.

Efficiency Of Different Therapeutic Methods On Virus Elimination (Dup: Abstract ?)

The influences of 30–40 mA current intensity, 1.0–2.0 mm cryo- and heat-treated shoot tips, and 25 mg L^{-1} ribavirin on ACLSV, ApMV and TRSV eradication were compared in three infected varieties (Table 5). The results revealed that virus elimination is prominently variety- and virus type-dependent. Thermotherapy of vars. Ordubad , Shams and Qaysi led to production of 90–100% virus-free plants and exhibited

superior rate of ACLSV, ApMV and TRSV eradication as compared to electro (40–60%)-, cryo (60–80%)- and chemo (75–100%)- therapies (Table 5). Virus eradication were confirmed by DAS-ELISA and RT-PCR tests (Fig. 4).

Table 5
Eradication rates of ACLSV, ApMV and TRSV using different therapeutic methods in three vars. of *P. armeniaca* L.

		ACLSV	ApMV	TRSV
Ordubad	Electro. 30–40 mA	47 ^{c*}	40 ^{c}	53 ^{c}
	Thermo. 1-2mm	90 ^{a}	90 ^{a}	100 ^{a}
	Chemo. 25 mg L^{-1} ribavirin	80 ^{b}	80 ^{b}	90 ^{b}
Shams	Electro. 30-40mA	60 ^{c}	53 ^{c}	47 ^{c}
	Thermo. 1-2mm	100 ^{a}	100 ^{a}	97 ^{a}
	Chemo. 25 mg L^{-1} ribavirin	90 ^{b}	80 ^{b}	75 ^{b}
Qaysi	Electro. 30-40mA	60 ^{d}	40 ^{c}	40 ^{c}
	Cryo. 1–2 mm	80 ^{c}	70 ^{b}	60 ^{b}
	Thermo. 1–2 mm	100 ^{a}	100 ^{a}	100 ^{a}
	Chemo. 25 mg L^{-1} ribavirin	90 ^{b}	100 ^{a}	100 ^{a}

* Within a column, means followed by the same letters are not significantly different according to DMRT at $P \leq 0.01$.

Discussion

Effect of different therapeutic methods on survival rate and survival of meristems

Viral diseases cause a general decline in the growth and productivity of fruit trees; therefore, production of virus-free materials is regarded as the very first crucial step prior to commercialization. Based on our results, electrotherapy of ELISA- and RT-PCR-positive samples with voltages more than 40 mA led to a drastic decrease in the survival rate of explants in all three varieties tested. This detrimental impact was more evident in var. 'Shams' as almost no shoots were regenerated with electric currents of 40–100 mA. Similar results have already been observed in other species like *Solanum tuberosum* L. (Meybodi et al. 2011) and *Narcissus tazetta* (Raj et al. 2022). The efficient electric current intensities for virus elimination followed by *in vitro* regeneration however seems to be species dependent as higher current intensities are required (40–100 mA) in *Vitis vinifera* L. (Guţă et al. 2019) to acquire regenerants free of virus.

Based on our results, unlike meristems sized between 0.2–0.7 mm which did not withstand droplet-vitrification cryotherapy, the 1.0–2.0 mm long explants were able to survive and regenerate. Shoot tips' excessive dehydration followed by cryotherapy causes adverse effects on explants' vitality. Due to the water crystallization inside the cells which causes rupture of cell membrane system, cell collapse and death, balancing dehydration step in success of this method is prominent (Wang et al. 2009). Generally, bigger meristems with more primordial leaves tend to have higher recovery rates and tolerance to liquid nitrogen treatment, yet accompanied with lower virus eradication rates (Zhao et al. 2018; Mathew et al. 2021). It is also claimed that only top layers of apical dome and primordial leaves can survive cryotherapy as non- or less- differentiated tissues with higher nucleo-cytoplasmic ratios are able to resist cryotherapy (Wang and Valkonen 2009). Our results also revealed that survival rate of shoot tips followed a genotype-dependent manner as the highest rates (10%) were attained in vars. 'Shams' and 'Qaysi' and the lowest (5%) was observed in var. Ordubad .

Axillary shoot multiplication from thermo-treated shoot tips also proved to be variety-dependent so that the highest number of shoots per explant (3.27) were achieved in var. 'Qaysi' followed by vars. 'Shams' (2.56) and 'Ordubad' (2.04), respectively. Similar results have already been detected in other species (reviewed by Wang et al. 2018). In pear (*Pyrus* spp.) for instance, amongst five varieties tested, the highest average of shoot proliferation was attained in var. "Abate Fetel" (1.227 shoots per explant), while the lowest number was observed in var. "Coscia" (0.638 shoots per explant) (Kazemi et al. 2020). According to our results, size of meristem exhibited a significant impact on survival rate of heat-treated apricot meristems and it raised with increasing size of explants. The lowest rates (6.66–13.33%) were attributed to 0.2–0.8 mm meristems while the highest (60.0–80.0%) were noted in 1.0–2.0 mm explants.

Synthetized in 1970, ribavirin (1-b-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) has been widely employed as an anti-viral agent in various animal and plant species (Thomas et al. 2012). In our study, inclusion of ribavirin in the culture medium led to drastic decrease in survival and multiplication rate of explants so that the highest rates (100% vitality and 2.33–4.08 shoots per explant) were attained in the control cultures (0 mg L^{-1}). Noxious effects of ribavirin on *in vitro* regeneration has already been reported in other species (Singh 2016; Guţă et al. 2017). Comparing the effects of ribavirin and 5-bromouracil in potato (*Solanum tuberosum*), Singh (2016) noted higher regeneration rate of explants in response to 5-Bromouracil as compared to ribavirin. Such a phytotoxic activity, however, seems to be species-dependent as no negative impacts have been reported in apples (Hu et al. 2022). In our study, supplementing culture medium with high doses exerted detrimental effects i.e., almost no vitality and proliferation were observed in all varieties tested upon exposure to 100 mg L^{-1} of ribavirin.

Efficiency Of Different Therapeutic Methods On Virus Elimination

Electrotherapy, the use of electrical currents to produce therapeutic effects, has long been applied on infected plant tissues for virus removal from the vegetative propagules (Nerway et al. 2020). In this study electrotherapy at 30–40 mA for 15 min, by which the highest rate of *in vitro* regeneration achieved, led to 40–53%, 47–60%, and 40–60% (depending on the type of virus) elimination in vars. Ordubad , Shams ,

and Qaysi , respectively. In this approach, virus elimination rates vary for different virus species depending on the intensity of electric current applied (reviewed by Adil et al. 2022). In *V. vinifera* L., *Iridaceae*, and *Asteraceae* family, application of 25–35 mA for 15–20 min was also recognized as the best treatment and resulted in 40% *Grapevine virus A* (GVA) (Bayati et al. 2011), 44–46% *Bean yellow mosaic virus* (BYMV) (Kaur et al. 2019), and 70–85% *Dahlia mosaic virus* (DMV) elimination (Nerway et al. 2020). In other species like *Solanum tuberosum* L. (Lozoya-Saldana et al. 1996; Mahfouze et al. 2010), however, lower current intensities (5–10 mA for 5–10 min) were required.

In the thermotherapy-based methods, infected *in vitro* cultures are subjected to heat for virus eradication. In this technique, the intensity of heat and duration of play crucial role and as a general rule, the higher the temperature and the longer the duration of exposure are, the higher rate of virus-elimination is (reviewed by Wang et al. 2018). According to our results, 90–100%, 97–100%, and 100% (depending on the type of virus) elimination were observed in the thermo-treated meristem tips of vars. Ordubad , Shams , and Qaysi , respectively.

In the present study, shoots were exposed to 24 ± 1°C in the first day and temperature increased 2˚C within a week reaching 38 ± 1˚C. Depending on virus type and plant species, as well as the virus-host combination, thermotherapy is generally conducted at 35–42°C for 4–6 weeks (Laimer et al. 2011; Panattoni et al. 2013; Barba et al. 2015). It has been postulated that viral RNA will be either silenced or degraded in leaves and shoot tips during thermotherapy possibly due to the cellular mechanism devoted to targeting and degradation of viral RNA at elevated temperatures (Wang et al. 2008). It is also noteworthy that, in order to be efficient, a thermotherapy regime should allow the treated explants to survive, on the one hand, and inactivate the virus, on the other.

Inclusion of 25 mg L^{-1} ribavirin in the culture medium also eradicated viral infection by 80–90%, 75–90%, and 90–100% (depending on the type of virus) in vars. Ordubad , Shams , and Qaysi , respectively. Antiviral property of ribavirin, either alone or in combination with other methods, has already been noticed against a broad spectrum of virus in a wide range of plant species (Yi et al. 2003; Ohta et al. 2011; Sedlák et al. 2019). Amongst five antiviral agents tested i.e., acycloguanosine, azidothymidine, 2,4-dioxohexahydro-1, 2,5-triazine (DHT), ribavirin and 2- thiouracil, against *Indian citrus ringspot virus* (ICRSV) in Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora), the maximum elimination rate (37%) was detected using 25 mg.L^{-1} ribavirin (Sharma et al. 2007). In *S. tuberosum* L., supplementing culture medium with 25 mg L^{-1} ribavirin also gave rise to 39.62% *Potato leaf roll virus* (PLRV)-free plants (Singh 2016). In spite of its proven effectiveness against a variety of viral agents, the exact underlying mechanism of ribavirin's action is still under debate. It has been suggested that binding of ribavirin in the form of ribavirin triphosphate (RTP) to the nucleotide binding site of the RNA-polymerase prevents the binding of the correct nucleotides, and thereby reduces viral replication or produces defective viruses (Te et al. 2007).

Conclusion

Viral diseases can cause drastic economic losses especially in vegetatively propagated fruit trees as they are more likely to be passed on to the offsprings. Hence, production of virus-free planting material is an

effective strategy to control viral diseases. In this study, amongst various methods tested, thermotherapy (38°C for 7 days) when applied to 1.0–2.0 mm explants let to 60–80% survival rate, the highest rate of virus eradication (90–100%), and therefore recognized as the most efficient viral elimination approach in apricot (*P. armeniaca* L.).

Declarations

Author contribution

RZ and MN conceived the idea and designed the experiments. AZK conducted the research; LM, MN, and BA assisted in data analysis and inscribing the manuscript. All authors discussed the results and contributed to the final manuscript.

Declaration of competing interest

Authors declare that they have no conflicting interests.

Data transparency

The data that support the findings of this study are openly available under reasonable request.

Research data policy and data availability statements

All data generated or analysed during this study are included in this published article.

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Figures

Figure 1 *In vitro* virus eradication in *Prunus armeniaca* L. **A:** shoots cut into 2-3 cm segments containing a single node, **B:** shoots transferred to the jars containing fresh modified QL medium after cryotherapy, **C:** shoot regeneration from thermo-treated explants, **D:** plantlets with well-developed shoots and roots, and **E:** acclimatized plantlet.

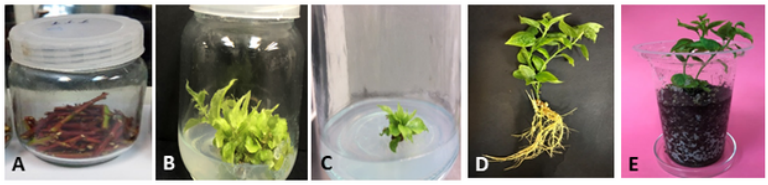

Figure 1

See image above for figure legend

Figure 2 Effect of electrotherapy voltages on the survival rate (%) of virus-infected *in vitro*-cultured shoots of three varieties of *P. armeniaca* L.

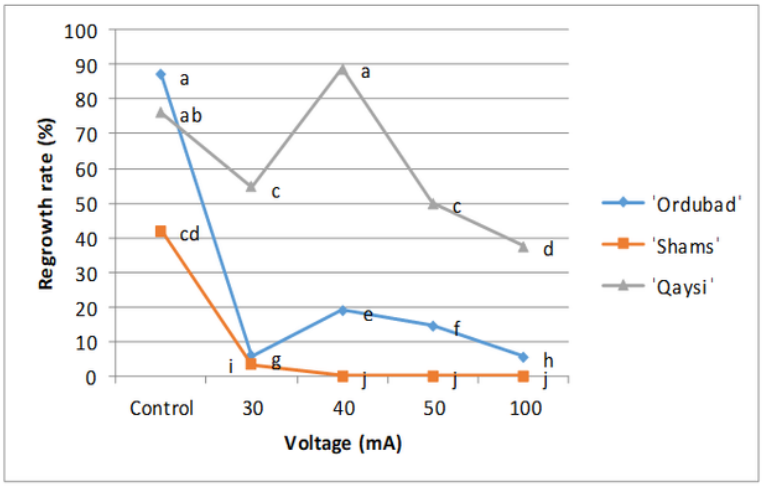

Figure 2

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Figure 3 The effect of thermotherapy regime on the regrowth rate (%) of different sizes of meristem in three vars. of *P. armeniaca* L.

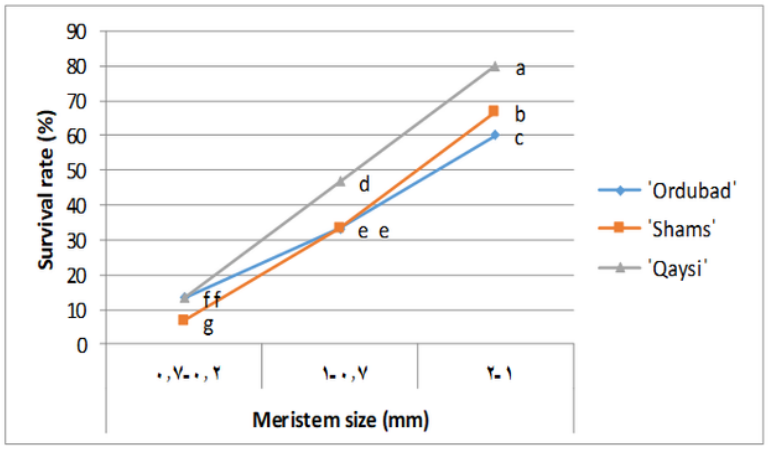

Figure 3

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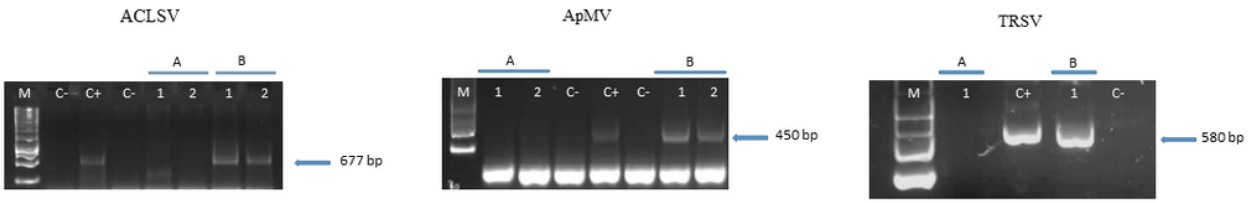
Figure 4 RT-PCR confirmation of ACLSV, ApMV, and TRSV elimination following eradication methods in *P. armeniaca* L.



A: after sanitation; **B:** prior to elimination; **C⁻:** negative control; **C⁺:** positive control; **M:** DNA marker 1 kb

Figure 4

See image above for figure legend