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Research Article

Developing virus-free grapevine explants by using silver-nanoparticles and its comparison with chemo and thermotherapy-based approaches

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Abstract: Grapevine viruses cause significant losses in the yield of grape. This study describes applying silver nanoparticles (AgNPs) to produce virus-free grapevine plants and compares it with chemo and thermotherapy. Preliminary molecular analysis proved the presence of *Grapevine fanleaf virus* (GFLV) and *grapevine leafroll-associated virus-1* (GLRaV-1) in the 'Asgari', 'Peykani', and 'Shahani' cultivar samples, then single node explants were cultivated in the MS medium. Thermotherapy at 35 ± 1 ºC and cycles of 35/38 ± 1 ºC, chemotherapy with ribavirin 0, 20, 25, and 30 µg.ml^{-1} and using AgNPs at 0, 10, 15, and 20 ppm in medium and 40, 50, and 60 ppm sprayed during acclimatization stage were applied to obtain virus-free explants. The results indicated that using 20 ppm AgNPs in medium and AgNPs combined treatment (15 ppm AgNPs in medium and sprayed with 50 ppm AgNPs in the acclimatization stage) were the most effective treatments for the elimination of viruses. The best treatment led to 100% eradication of GLRaV-1 and 67% of GFLV in 'Asgari', 100% eradication of GLRaV-1 and GFLV in 'Peykani' and 100% eradication of GLRaV-1 and 67% of GFLV in 'Shahani'. Furthermore, applying of AgNPs improved plant growth parameters, including plant height, which in infected plantlets was (18.06, 12.36, and 14.92 cm in 'Asgari', 'Peykani', and 'Shahani', respectively) less than virus-free plantlets. Leaf number was 45, 34, and 27 in virus-free plantlets of 'Asgari', 'Peykani', and 'Shahani', respectively, but in infected plantlets, it was 24.40, 19.80, and 12. Leaf area increased from 5.34, 5.50, and 5.94 cm^2 in infected plantlets to 9.56, 11.43, and 12.33 cm^2 in virus-free plantlets of 'Asgari', 'Peykani', and 'Shahani', respectively. Complementary results proved that chlorophyll content in virus-free is significantly higher than in virus-infected plantlets, which explains and confirms the change in growth parameters after virus removal.

Keywords: Grapevine, GLRaV-1, GFLV, nanoparticle, AgNPs

Introduction

Grapes are one of the main fruits used, whether fresh, fermented, or processed, and the second largest fruit, in quantity, grown in the world after oranges (Kumar and Prabhavathi, 2022). Plant viruses cause economic losses in grape production worldwide (Moradi *et al.*, 2017; Xiao

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et al., 2018). *Grapevine fanleaf virus* (GFLV) and *Grapevine leafroll-associated virus* (GLRaV) are among the most destructive grape viruses (Martelli, 2017, Meng *et al.*, 2017; Rodríguez-Verástegui *et al.*, 2022). GFLV and GLRaV influence the plant's growth and environmental responses, including a reduction in plant vigor, degeneration and malformations of leaves, shoots, and clusters, and yield and fruit quality leading to significant economic losses in the whole grapevine agribusiness (Rienth *et al.*, 2021). Therefore, producing virus-free plants improves the quantity and quality of grapevine crop production (Yan *et al*., 2022).

Standard techniques for obtaining virus-free grapevine plants are meristem tip culture, somatic embryogenesis, chemotherapy, thermotherapy, electrotherapy, shoot tip cryotherapy, and micro grafting (Hu *et al*., 2021; Yan *et al*., 2022). However, according to many researchers, chemotherapy is the most effective technique compared to other methods because by achieving antiviral chemical compounds that have been identified against the replication cycles of viruses, this cycle can be disrupted (Panattoni *et al*., 2007). Some enzymes are involved in the biosynthesis of virus nucleotides, which synthesize DNA and RNA and eventually amplify these pathogens. The most important enzyme is the inosine-5'-monophosphate dehydrogenase (IMPDH) enzyme, which has been found to cure many viral diseases, even in medical research, by inhibiting IMPDH (Cuny *et al*., 2017). IMPDH is involved in the biosynthesis of guanine nucleotides, and inhibitors of this enzyme activate a mechanism to interrupt the synthesis of viral DNA and RNA by blocking the conversion pathway of inosine-5'-monophosphate (IMP) to Xanthosine-5'-monophosphate (XMP) (Hedstrom, 2009). S-Adenosyl-L-homocysteine (SAH) is another significant enzyme in the maturation cycle of viral mRNAs that has always been a main molecular target for antiviral programs alongside IMDPH (De Clercq, 2004). The most widely used viral inhibitor is ribavirin, which prevents the elongation of virus mRNA and, consequently, its nucleic acid synthesis by its multifaceted function (Leyssen *et al*., 2005). This antiviral agent has successfully eradicated up to 80% of viruses in some plants, including apples and pears (Hu *et al*., 2012; Hu *et al*., 2015). However, there are other antivirals that, alone or in combination with ribavirin or thermotherapy, may lead to the elimination of the virus (Carvalho *et al*., 2022). In recent years, nanoparticles have become particularly important in treating and preventing diseases in the field of medicine (Cai *et al*., 2019). The triumphant performance of nanoparticles in medicine has also provided the basis for their application in agriculture (Tripathi *et al*., 2018). Using metal nanoparticles could be a new opportunity to control plant viral diseases. Because the main mechanisms of nanoparticles action which will inhibit the activity of viruses include: interaction with glycoprotein (gp120), competition for virus binding to the cell, inactivation of virus particles before entry and binding to viral particles (Galdiero *et al*., 2011; Vargas-Hernandez *et al*., 2020). Studies on Solanaceae, Asteraceae, *Poaceae, Fabaceae, and Cucurbitaceae* express inhibition of virus replication by penetration of MeNPs into the vascular system (Elazzazy *et al*., 2017; Shafie *et al*., 2018; Cai *et al*., 2020). However, no research has been done on the effect of MeNPs to control grape viruses and compare the routine methods of virus removal (chemo and thermotherapy) with nanoparticles. To that end, due to the nutritional and economic crucial importance of grapes, the role of viruses in reducing their yield, and the lack of a method with a high response rate in controlling these pathogens, in this study, nanoparticles were used to control the important grape viruses in tissue culture.

Materials and Methods

Plant materials

Grape buds of the Iranian cultivars, including 'Asgari', 'Peykani', and 'Shahani' were collected from the grape germplasm collection orchard of Agricultural and Natural Resources Research and Education Center of Shahrood and Kashmar, Semnan and Khorasan provinces in Iran. Firstly buds were washed with tap water and disinfected with ethanol 70% (1 min) and sodium hypochlorite solution 3.5% (12 min). The single

node explants were cultivated in the MS medium (Murashige and Skoog 1962), supplemented with 1 $mg.l^{-1}$ 6-Benzylaminopurine (BAP), 30 $g.l^{-1}$ sucrose, and eight $g.l^{-1}$ agar (Merck) (Hu *et al.*, 2018). All plantlets were incubated at 24 °C with a 16-h light/8-h dark photoperiod (2000 lx light intensity). After one subculture (45 days later) in the same medium, the uniform and identical established shoots (3 cm length) were used for GLRaV-1 and GFLV detection; if the samples were infected, they were used to apply antiviral treatments.

Thermotherapy

Infected shoots (3 cm) were cultured in MS medium supplemented with 1 $mg.l^{-1}$ BAP and 0.05 $mg.l^{-1}$ IBA. Five days after culture, two methods were performed: 1) Constant temperature 35 ± 1 ºC, 16/8 h light/dark photoperiod (the temperature was gradually increased to 35 °C during three days). 2) Alternating 6-hr. cycles of 35/38 ± 1 ºC on a 16/8 h light/dark photoperiod (the temperature was gradually increased to 35 °C during three days, then a temperature cycle was applied). Meristems (containing two or three leaf primordia; < 0.5 mm length) of these shoots were dissected after incubation for 20 days. All treated meristems were incubated on a modified MS medium with 0.5 $mg.l^{-1}$ BAP, 0.05 $mg.l^{-1}$ indole-3-butyric acid (IBA), 20 $g.l^{-1}$ sucrose, and 8 $g.l^{1}$ agar.

Chemotherapy

Ribavirin (Virazole; Sigma-Aldrich) was filter-sterilized with a 0.22 µm Millipore filter and added to the fresh MS medium (1 $mg.l^{-1}$ BAP and 0.05 $mg.l^{-1}$ IBA, 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar) inside a laminar flow at final concentrations of 20, 25 and 30 $\mu g.ml^{-1}$. The grapevine shoots were transferred to this medium. All treated samples were incubated in a tissue culture room with the above conditions for 45 days.

Nanoparticles

Silver nanoparticles (AgNPs) of 15 nm size, in liquid form, were purchased from Sigma Company, and three methods were performed to apply AgNPs (nano-control). 1) AgNPs were added to the fresh MS medium (1 $mg.l^{-1}$ BAP and 0.05 $mg.l^{-1}$ IBA, 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar) inside a laminar flow at final concentrations of 10, 15 and 20 ppm. 2) Plantlets that were in the acclimatization stage after rooting were sprayed with 40, 50, and 60 ppm AgNPs 15 days after the acclimatization stage in two 10-day periods; 3) Combination of the above two treatments (15 ppm AgNPs in medium and sprayed with 50 ppm AgNPs in the acclimatization stage). All treated samples were incubated in a tissue culture room with the above conditions for 45 days.

Furthermore, grapevine plants from the same source were used as controls, and 20 replications of five explants were used in all treatments.

Determination of chlorophyll content and physiological factors

Each experiment was performed in a separate completely randomized design (CRD) with 20 replications and five plants for each replication (Fig. 1). First, shoot-tip necrosis, successful meristem establishment, and incidence of hyperhydricity were evaluated in all treatments to investigate the effect of each on seedling viability. Then a total of 40 rooted micro-propagated plants were evaluated in each treatment. After three months, 20 plants from each treatment were used to record plant length (cm), leaf number, leaf area (cm^2), and chlorophyll in the greenhouse. The leaf area of the four oldest leaves of each plant was measured using a Li-Cor 1300 area meter (Li-Cor Biosciences, Lincoln, NE, USA). Chlorophyll content was recorded using a Minolta SPAD-502 meter, a non-destructive measuring device. SPSS was used for statistical analysis of physiological and SPAD-502 measurements, and means were compared based on the LSD test at 5% probability level.

Detection of viruses by PCR analysis

Presence of GFLV and GLRaV in plant materials before and three months after treatment of the explants was assessed by PCR analysis. For this purpose, total RNA was extracted from 200 mg of young leaves as described by Hu *et al.* (2015).

cDNA was synthesized from 1 µg of total RNA using the RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The forward and reverse primer sequences in Table 1 were used for amplification. The PCR amplification was carried out in a Variti 96-well thermal cycler using the following cycles:

GLRaV-1: initial denaturing at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s, extension at 72 °C for 60 s, and the final extension at 72 °C for 5 min.

GFLV: initial denaturing at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 45 s, extension at 72 °C for 80 s, and the final extension at 72 °C for 10 min.

The PCR products were electrophoresed on 1.5% agarose gel in TAE buffer stained with ethidium bromide and then visualized under UV light (Gelduc., UK). The size of the amplified fragments was determined using a Gen Ruler 1 kb plus DNA Ladder (Thermo Scientific, USA).

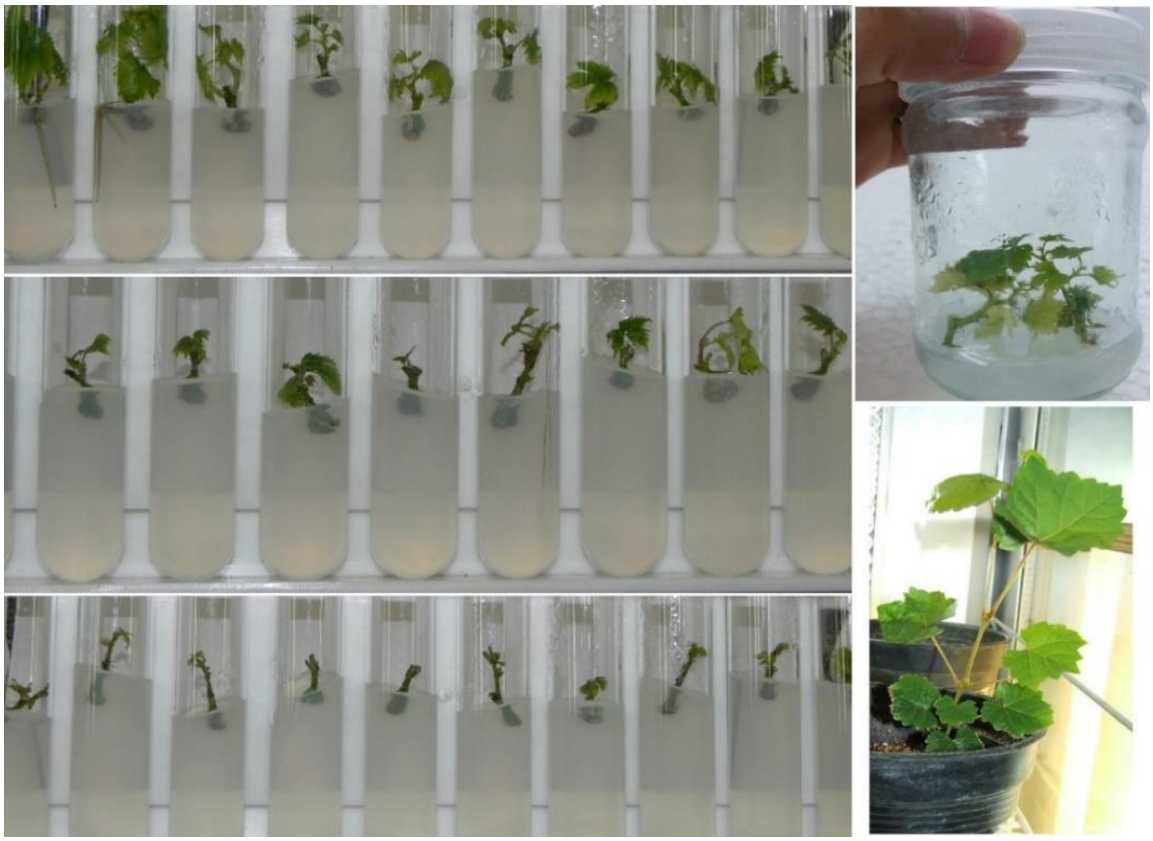

Figure 1 Illustrations of growth stages in the production of in vitro grapevine plantlets following the thermotherapy, chemotherapy with ribavirin and AgNPs, and the combined treatment.

Table 1 Primer pairs used for detecting GLRaV-1 and GFLV in grapevine plantlets.

Target	PCR product size (bp)	Primer names	Nucleotide sequences from 5' to 3'
GLRaV-1	398	LEV1-C447	CGACCCCTTTATTGTTTGAGTATG
		LQV1-H47	GTTACGGCCCTTTGTTTATTATGG
GFLV	1000	DetF	CGGCAGACTGGCAAGCTGT
		DetR	GGTCCAGTTTAATTGCCATCCA

Results

Plantlets viability

The main issue in eradicating plant viruses is the survival of the plantlets until the final stages of obtaining virus-free plants. Therefore, plant durability, including shoot-tip necrosis, successful meristem establishment, and hyperhydricity parameters (in % of number of plants treated) were measured to determine the survival rate of treated plantlets (Table 2). As expected, the treatments had different efficacy on shoot-tip necrosis, successful meristem establishment, and hyperhydricity. Some treatments caused the complete demolition of plantlets, and some weakened them. Thermotherapy (cycles of 35/38 ± 1 ºC) had the most prominent and destructive effect on these parameters. It decreased the survival rate of treated plantlets, which were necrotic and annihilated up to 56, 63.20 and 42.20% of plantlets in 'Asgari', 'Peykani' and 'Shahani' cultivars, respectively. It is noteworthy that in 'Shahani' cultivar, a red grape, the survival rate of plantlets was higher than the other two cultivars. In 'Shahani' cultivar, shoot-tip necrosis was 13.80 and 21.00% lower than 'Asgari' and 'Peykani', respectively. Also, successful meristem establishment in this cultivar was 12.20 and 7.80% higher than ' respec and 'Peykani', respectively. The trend of hyperhydricity was similar to necrosis, and in 'Shahani' cultivar was 4.60 and 5.80% lower than 'Asgari' and 'Peykani', respectively. Thickness of leaves can be one of the main reasons for the loss of grape plantlets in the thermotherapy period because evaporation and transpiration is higher in thin leaves; as a result, they are more sensitive to rising temperatures.

In chemotherapy and utilization of nanoparticles, the three cultivars showed almost the same response in viability parameters. The survival rate in these treatments was higher than that of thermotherapy, but with increasing ribavirin and AgNPs concentration, necrosis and hyperhydricity increased in all three cultivars. Results showed 'Shahani' cultivar was still less sensitive to high concentrations of these substances. At a concentration of 30 µg.ml-1 ribavirin, the rate of hyperhydricity in 'Asgari' and 'Peykani' cultivars reached 29.00% and 25.00%, respectively, while it was 17.20% in 'Shahani' at the same concentration of ribavirin.

Increasing the AgNPs concentration in the culture medium also caused a decreasing effect on plantlets viability but not as much as ribavirin, which could indicate a better result than ribavirin if combined with the impact of AgNPs on virus elimination. So the successful meristem establishment in a high concentration of AgNPs (20ppm) was 52.80, 50.80, and 59.40% 'Asgari', 'Peykani' and 'Shahani', respectively. In AgNPs spray treatments, plantlet durability parameters were similar to controls in all three cultivars because the plantlets were treated in the acclimatization stage (Table 2).

The efficiency of GLRaV-1 and GFLV eradication by thermotherapy, ribavirin, and AgNPs

The results of different procedures for eradicating GLRaV-1 and GFLV are given in Table 3. The RT-PCR results for GLRaV-1 and GFLV detection showed that infected plantlets of all three cultivars under 35 ± 1 ºC temperature remained still infected; on the other hand, the cycle of 35/38 ± 1 ºC in 'Asgari' and 'Peykani' performed better than 'Shahani' plantlets. According to RT-PCR results, after this temperature cycle, 66% of plantlets were free of GLRaV-1 and 33% free of GFLV in both 'Asgari' and 'Peykani' cultivars. But about 'Shahani' cultivar, these percentages were reduced to 33 (GLRaV-1) and zero (GFLV). Plantlets that survived at high ribavirin concentrations were GLRaV-1-free (66%) and GFLV-free (33%), but the presence of these two viruses was still confirmed in 'Peykani' and 'Shahani' after three months.

As mentioned above, the application of AgNPs performed well in plantlet survival. It was also more successful than thermotherapy and ribavirin in virus eradication. AgNPs at high concentrations (20 ppm) eradicated GLRaV-1 and GFLV viruses from 66% of the plantlets in all cultivars. Virus eradication reached its peak when AgNPs combined treatment (15 ppm AgNPs in culture medium and sprayed with 50 ppm AgNPs at the acclimatization stage) was used. After the mentioned treatment, all three cultivars were 100% GLRaV-1-free, and 'Peykani'and 'Shahani'66% were GFLV-free (Table 3).

Table 2 The effect of treatments on plant durability, including shoot-tip necrosis (A), successful meristem establishment (B), and hyperhydricity (C), in % of number of a plants treated.

Treatment		'Asgari'			'Peykani'			'Shahani'		
		A	B	C	A	B	C	A	B	C
Control	Without treatment	3.80^{ijk}	75.40^{a}	5.20^{g}	3.20^{h}	68.60^{bc}	6.00^{f}	1.00^{g}	77.80^{a}	8.20^{cd}
Thermotherapy	35 ± 1 ºC	46.60^{b}	48.20^{f}	12.20^{d}	52.20^{b}	45.00^{h}	8.80^{de}	32.80^{b}	58.20^{ef}	6.80^{cdef}
	cycles of 35/38 ± 1 ºC	56.00^{a}	31.60^{h}	9.40^{de}	63.20^{a}	36.00^{i}	10.60^{d}	42.20^{a}	43.80^{g}	4.80^{efg}
Ribavirin	20 µg.ml^{-1}	22.80^{d}	61.60^{d}	4.80^{g}	21.60^{d}	61.20^{ef}	7.20^{ef}	10.40^{c}	73.40^{bc}	4.20^{fg}
	25 µg.ml^{-1}	19.20^{e}	55.80^{e}	10.80^{de}	14.00^{e}	48.80^{g}	16.20^{bc}	12.00^{c}	68.00^{d}	9.00^{c}
	30 µg.ml^{-1}	24.80^{c}	37.00^{g}	29.00^{a}	27.80^{c}	36.20^{i}	25.00^{a}	10.80^{c}	54.40^{f}	17.20^{a}
AgNPs in medium	10 ppm	4.80^{ij}	68.20^{c}	9.00^{ef}	2.20^{h}	71.00^{ab}	6.80^{ef}	3.00^{efg}	75.80^{ab}	3.20^{g}
	15 ppm	7.20^{h}	64.00^{d}	18.60^{bc}	11.80^{ef}	58.40^{f}	14.40^{c}	4.80^{de}	71.20^{cd}	7.00^{cde}
	20 ppm	16.00^{f}	52.80^{e}	21.00^{b}	9.40^{fg}	50.80^{g}	17.80^{b}	5.40^{d}	59.40^{e}	11.80^{b}
AgNPs spray	40 ppm	2.00^{k}	73.80^{ab}	3.80^{g}	4.00^{h}	71.20^{ab}	7.20^{ef}	2.20^{fg}	79.00^{a}	6.00^{def}
	50 ppm	5.20^{i}	69.80^{c}	6.00^{fg}	3.00^{h}	72.80^{a}	4.60^{f}	4.20^{def}	76.20^{ab}	4.200^{fg}
	60 ppm	3.20^{jk}	71.20^{bc}	5.80^{g}	4.20^{h}	66.80^{cd}	5.20^{f}	3.40^{def}	73.00^{bc}	5.20^{efg}
AgNPs combined treatment	15 ppm (medium) + 50 ppm (spray)	12.00^{g}	61.80^{d}	15.80^{c}	8.60^{g}	63.40^{de}	11.20^{d}	4.00^{def}	72.60^{bc}	6.40^{cdef}

Table 3 Efficiency of thermotherapy, ribavirin and AgNPs on GLRaV-1 and GFLV eradication from grapevine plantlets ('Asgari', 'Peykani' and 'Shahani' cultivars) using RT-PCR.

Treatment		'Asgari'		'Peykani'		'Shahani'	
		number of virus-free/ tested plants		number of virus-free/ tested plants		number of virus-free/ tested plants	
		GLRaV-1	GFLV	GLRaV-1	GFLV	GLRaV-1	GFLV
Control	Without treatment	0/3	0/3	0/3	0/3	0/3	0/3
Thermotherapy	35 ± 1 ºC	0/3	0/3	0/3	0/3	0/3	0/3
	cycles of 35/38 ± 1 ºC	2/3	1/3	2/3	1/3	1/3	0/3
Ribavirin	20 µg.ml^{-1}	0/3	0/3	0/3	0/3	0/3	0/3
	25 µg.ml^{-1}	0/3	0/3	0/3	0/3	0/3	0/3
	30 µg.ml^{-1}	2/3	1/3	1/3	0/3	0/3	1/3
AgNPs in medium	10 ppm	0/3	0/3	0/3	0/3	0/3	0/3
	15 ppm	2/3	1/3	2/3	0/3	1/3	0/3
	20 ppm	2/3	2/3	3/3	2/3	2/3	1/3
AgNPs spray	40 ppm	0/3	0/3	0/3	0/3	0/3	0/3
	50 ppm	1/3	0/3	1/3	1/3	0/3	0/3
	60 ppm	1/3	1/3	2/3	0/3	1/3	1/3
AgNPs combined treatment	15 ppm (medium) + 50 ppm (spray)	3/3	2/3	3/3	3/3	3/3	2/3

Effect of virus eradication on the growth of plantlets and Chlorophyll content

Different treatments which led to virus eradication caused a significant increase in the growth parameters (plant length, leaf number, and leaf area) compared to GLRaV-1 and GFLV-infected grapevine plants (controls) in each of the three cultivars. The highest growth parameters were observed in AgNPs treatments (Table 4). Because these treatments are more effective in virus eradication, and also AgNPs increase the absorption of nutrients and directly affect plant growth. According to the results, the maximum plant length was reported in 20 ppm AgNPs and AgNPs combined treatment (15 ppm (medium) + 50 ppm (spray)). Plant length in untreated and infected plantlets was lower (18.06, 12.36, and 14.92 cm in 'Asgari', 'Peykani', and 'Shahani', respectively) than GLRaV-1-free and GFLV-free plantlets. The same was reported about leaf number and leaf area (cm^2).

Leaf number and leaf area in virus-infected plants were much lower than in treated virus-free plants(especially AgNPs treatments). The maximum leaf number was reported in AgNPs combined treatment which were 45, 34, and 27 in

virus-free plantlets of 'Asgari', 'Peykani', and 'Shahani' cultivars, respectively, but in infected plantlets, it was 24.40, 19.80 and 12, respectively. Also, leaf area increased from 5.34, 5.50, and 5.94 (cm^2) in infected plantlets to 9.56, 11.43, and 12.33 (cm^2) in virus-free plantlets of 'Asgari', 'Peykani', and 'Shahani' cultivars, respectively. This could indicate the effect of the virus's presence on reducing the growth of the studied cultivars.

Also SPAD readings of total chlorophyll content in treatment were significantly different for virus-infected and virus-free plantlets (Fig. 2). Total chlorophyll content in untreated and infected plantlets was lower than GLRaV-1-free and GFLV-free plantlets after three months of greenhouse acclimatization; which compared to infected plantlets confirms that photosynthetic rate is affected by GLRaV-1 and GFLV through decreasing leaf chlorophyll content. The highest amount of chlorophyll was observed in the AgNPs combined treatment, which also had the greatest effect on virus eradication.

Table 4 The growth parameters of grape plants treated with different treatments, compared with control.

Treatment		'Asgari'			'Peykani'			'Shahani'		
		PL	LN	LA	PL	LN	LA	PL	LN	LA
Control	Without treatment	18.00^{gh}	24.40^{j}	5.34^{ef}	16.98^{g}	19.80^{fg}	5.50^{i}	14.22^{f}	12.00^{hi}	5.94^{i}
Thermotherapy	35 ± 1 ºC	18.96^{g}	26.20^{ij}	5.20^{ef}	15.10^{h}	17.00^{h}	5.63^{hi}	16.10^{ef}	10.00^{i}	6.32^{hi}
	cycles of 35/38 ± 1 ºC	28.18^{d}	32.00^{f}	8.42^{b}	24.00^{c}	27.20^{c}	10.30^{b}	21.14^{c}	19.40^{cde}	7.73^{d}
Ribavirin	20 µg.ml^{-1}	17.28^{h}	28.00^{hi}	4.93^{f}	16.08^{gh}	19.00^{gh}	5.33^{i}	15.10^{ef}	14.00^{gh}	6.09^{i}
	25 µg.ml^{-1}	18.94^{g}	29.00^{gh}	5.71^{de}	17.16^{g}	18.80^{gh}	6.15^{gh}	17.00^{e}	15.20^{fg}	6.30^{hi}
	30 µg.ml^{-1}	26.00^{e}	35.00^{e}	9.08^{a}	19.06^{f}	21.80^{def}	8.66^{c}	22.02^{c}	21.00^{c}	7.41^{de}
AgNPs in medium	10 ppm	21.08^{f}	31.00^{fg}	6.15^{d}	19.00^{f}	20.00^{fg}	6.90^{ef}	19.06^{d}	18.00^{de}	6.91^{fg}
	15 ppm	28.12^{d}	38.20^{cd}	8.05^{bc}	22.18^{d}	23.20^{de}	7.41^{e}	21.06^{c}	20.00^{cd}	7.81^{d}
	20 ppm	36.06^{a}	42.00^{b}	9.28^{a}	26.10^{b}	31.00^{b}	10.92^{a}	26.14^{b}	26.00^{ab}	12.33^{a}
AgNPs spray	40 ppm	21.92^{f}	32.00^{f}	5.95^{d}	21.00^{de}	21.00^{efg}	6.21^{g}	21.06^{c}	17.20^{ef}	6.65^{gh}
	50 ppm	29.10^{cd}	37.20^{de}	6.28^{d}	20.10^{ef}	23.00^{de}	6.81^{f}	22.98^{c}	18.40^{de}	7.14^{ef}
	60 ppm	30.06^{c}	40.00^{bc}	7.73^{c}	24.08^{c}	23.80^{d}	8.11^{d}	25.84^{b}	24.20^{b}	8.30^{c}
AgNPs com bined treatment	15 ppm (medium) + 50 ppm (spray)	34.20^{b}	45.00^{a}	9.56^{a}	29.34^{a}	34.00^{a}	11.43^{a}	29.14^{a}	27.00^{a}	11.18^{b}

PL: plant length (cm); LN: leaf number; LA: leaf area (cm^2).

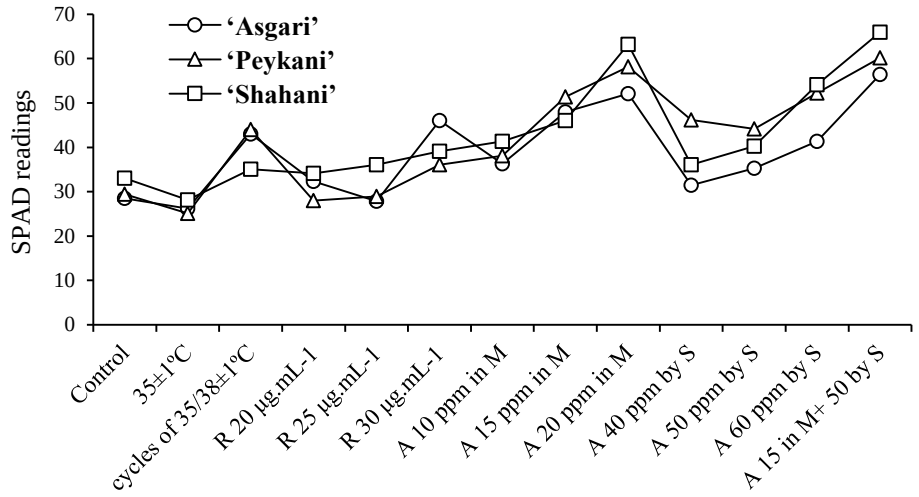

Figure 2 SPAD readings of grape leave in different treatments after three months in the greenhouse.

Discussion

In recent years, research has been conducted to remove plant viruses using thermotherapy, chemotherapy, and nanoparticles. But in grapevines, a comprehensive and comparative study has not been done on virus eradication by different methods, especially in different cultivars. In the current research, common methods have been used to remove viruses in addition to nanoparticles. Each method performed in each cultivar showed different effects on virus removal, plantlet survival, and growth. As stated in various research, high temperatures cause stress in plants' developmental stages, and such stress is intensified as temperature increases (Wahid *et al.*, 2007; Wang *et al.*, 2018).

On the other hand, the higher the temperature and the longer the duration of exposure, the higher the frequency of virus eradication (Panattoni *et al.*, 2013; Barba *et al.*, 2015; Wang *et al.*, 2018). However, high temperatures and prolonged duration have reduced the survival rate of treated shoots and the ability to regenerate the tips of shoots separated from treated plantlets (Hu *et al.*, 2015; Zhao *et al.*, 2018). Thus high temperature affected the viability of plantlets, and the effect of high temperature on these parameters was different in each cultivar. Current research showed that 'Shahani' cultivar was more resistant to temperature due to thicker leaves. After cycles of 35/38 ± 1 ºC 33% of 'Shahani' plantlets were free of GLRaV-1 and still infected with GFLV (Table 3).

It should be noted that plant survival and virus eradication depend mainly on the type of virus, plant species, their cultivars, and the virus-host composition (Laimer and Barba, 2011; Barba *et al.*, 2015). In this regard, the use of alternating temperatures showed positive effects in reducing the adverse effects of high constant temperatures on plantlets during thermotherapy and increasing the survival and growth of heat-treated shoots and thus improving the eradication efficiency of the virus (Wang and Valkonen, 2009; Karimpour *et al.*, 2021). The same results were achieved in apple and pear, indicating that applying thermal cycles was an effective thermotherapy treatment to eradicate the virus (Tan *et al.*, 2010; Karimpour *et al.*, 2021).

The results of ribavirin in line with thermotherapy showed that the success rate of removal of viruses depends on the plant cultivar and the type of virus (Barba *et al.*, 2015). In contrast to thermotherapy, ribavirin in various plants like Lebanese fig varieties (Chalak *et al.*, 2015) and grapevine (Komínek *et al.*, 2016). has been able to incorporate into RNA during viral replication and lead to lethal mutations in viruses (Hu *et al.*, 2021). According to the current study, it was perceived that ribavirin eradicated the virus in grapevine cultivars, but plantlet viability decreased at higher concentrations (30 $\mu g.ml^{-1}$). On the other hand, 'Shahani' cultivars were still infected even after high concentrations of ribavirin (Table 3).

The success rate of nanoparticles in virus eradication can also be affected by the cultivar and type of virus. Still, as various studies have demonstrated that nanoparticles performed better than thermotherapy and ribavirin in virus eradication (Hill and Whitham, 2014), the results of this study also confirmed the same. Results of EI Gamal *et al.* (2022) suggest that AgNPs have curative viricidal activity due to targeting the virus coat protein and affecting virus-vector interactions. So that the high concentration of AgNPs caused the eradication of both viruses in 'Asgari' cultivar, and combined treatment including AgNPs in culture medium and spraying it in the acclimatization stage showed a successful result in 'Shahani' and 'Peykani' cultivars. So it can be said that combined treatment has a better efficacy, indicating the effect of AgNPs on different stages of plant growth (Vargas-Hernandez *et al.*, 2020). In *Chenopodium amaranticolor* plants decrease in virus concentration and disease percentage after AgNPs spray against tomato spotted wilt virus (TSWV) (Shafie *et al.*, 2018) and potato plants against tomato bushy stunt virus (TBSV) (El-shazly *et al.*, 2017) was obtained. The results of this study showed that the total elimination rates of GLRaV-1 were more than that of GFLV,

suggesting that GLRaV-1 was eradicated more easily than GFLV.

To some extent, the elimination rate of GLRaV-1 could be a good indicator of its weakness compared to GFLV. Because the applied MeNPs interfere mainly during the early virus replication cycle through numerous mechanisms, each virus has a different replication process. According to the studies conducted, the underlying mechanisms are the inactivation and denaturation of capsid protein, nucleic acids (RNA or DNA), and other protein molecules. The MeNPs may also prevent virion binding, fusion, infectivity, and replication (Dutta *et al.*, 2022). Therefore virus eradication can be affected by virus' molecular structure, morphology, and the particular proteins specific to each virus (Hu *et al.*, 2018; Hu *et al.*, 2021).

In the current investigation, eradication of GLRaV-1 and GFLV after treatment with different methods recorded a significant increase in the growth of grapevine plantlets. After three months, treatments that eradicated the virus substantially increased plant lengths, leaf number, leaf area, and chlorophyll content. As Mahfouze *et al*. (2020) reported, virus-infected banana plants treated with 50 ppm AgNPs significantly increased growth factors and changes in chlorophyll (a and b). Also, Salama (2012) observed that using AgNPs led to a significant increase in shoot and root lengths, leaf area, and chlorophyll in *Zea mays* L. and *Phaseolus vulgaris* L. plants. On the other hand, shoot-tip necrosis and hyperhydricity are the most common problems in thermotherapy, and high concentrations of ribavirin cause a significant loss of virus-free plants (Karimpour *et al.*, 2021). Present results showed that high temperatures and high concentrations of ribavirin caused shoot-tip necrosis and decreased shoot-tip survival, which was in line with the hyperhydricity of explants. Shoot-tip necrosis due to high concentration of ribavirin and high temperature has been reported in grapevines (Hu *et al.*, 2021) and other plants, including apples (Karimpour *et al.*, 2021). However, the application of AgNPs reduced shoot-tip necrosis and hyperhydricity, and the survival percentage of explants increased significantly compared to the other two methods (chemo and thermotherapy). Therefore, according to the present results, nanoparticles performed better than the other two methods in eradicating viruses, increasing growth parameters, and also plantlets' survival. Also, considering that the use of MeNPs in *invitro* conditions is much easier than thermotherapy, this method can be proposed as an optimized approach and routine technique for producing virus-free grapevines.

The rate of development and application of antiviral agents is promising. One of them is nanoparticles which is a profitable opportunity for novel antiviral therapy uses in agriculture; nevertheless, it is essential to take into account their toxicity and the application of nanoparticle doses (Hoseinzadeh *et al*., 2016). But certain concentrations which showed antiviral activity do not have negative impacts on plants and toxicity to humans (Vargas-Hernandez *et al*., 2020).

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بهینه‌سازی تولید ریزنمونه‌های انگور بدون ویروس با استفاده از نانوذرات نقره و مقایسه آن با روش‌های گرمادرمانی و شیمی درمانی

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چکیده: با توجه به تأثیر عمده ویروس‌های انگور بر عملکرد این محصول، مطالعه حاضر کاربرد نانوذرات نقره برای تولید گیاه انگور عاری از ویروس را بررسی کرده و با شیمی‌درمانی و حرارت درمانی مقایسه می‌شود. تجزیه و تحلیل اولیه وجود ویروس برگ بادبزنی انگور (GFLV) و ویروس پیچیدگی برگ انگور (GLRaV-1) را در نمونه‌های ارقام عسگری، پیکانی و شاهانی ثابت کرد. سپس ریزنمونه‌های هر رقم به‌صورت جداگانه در محیط کشت MS کشت شدند. برای به‌دست آوردن ریزنمونه‌های عاری از ویروس، گرمادرمانی (۱ ± ۳۵ درجه سلسیوس و سیکل‌های ۱ ± ۳۵/۳۸ درجه سلسیوس)، شیمی‌درمانی (ریباویرین صفر، ۲۰، ۲۵ و ۳۰ میکروگرم در میلی‌لیتر) و استفاده از نانوذرات نقره (AgNPs) (صفر، ۱۰، ۱۵ و ۲۰ پی‌پی‌ام در محیط و ۴۰، ۵۰ و ۶۰ به‌صورت محلول‌پاشی در مرحله سازگاری گیاهچه‌ها) استفاده شد. نتایج نشان داد میزان ریشه‌کنی ویروس در رقم شاهانی کم‌تر از دو رقم دیگر بود. هم‌چنین نتایج حاکی از عملکرد بهتر نانوذره نقره نسبت به حرارت درمانی و ریباویرین در حذف ویروس و افزایش فاکتورهای رشدی گیاه بود. کاربرد ۲۰ پی‌پی‌ام نانوذرات نقره در محیط کشت و تیمار ترکیبی ۱۵ پی‌پی‌ام نانوذره نقره در محیط و محلول‌پاشی با ۵۰ پی‌پی‌ام نانوذرات نقره در مرحله سازگاری مؤثرترین تیمار در هر سه رقم بود. هم‌چنین میزان کلروفیل کل تفاوت معنی‌داری را بین هر سه رقم نشان داد، به‌طوری‌که افزایش میزان کلروفیل در گیاهچه‌های آلوده به ویروس پس از اعمال تیمارها و مقایسه آن با گیاهچه‌های بدون ویروس مشهود بود که تغییر در فاکتورهای رشدی گیاه پس از حذف ویروس را نیز تأیید می‌کند.

واژگان کلیدی: انگور، ویروس پیچیدگی برگ انگور، ویروس برگ بادبزنی انگور، نانوذرات، نانوذرات نقره