Pak. J. Bot., 58(8) August 2026.

ARTICLE | DOI: http://dx.doi.org/10.30848/PJB2026-8(14)

DETECTION AND *GRAPEVINE YELLOW SPECKLE VIROID 2* ELIMINATION IN PAKISTANI GRAPEVINES THROUGH MERISTEM TIP CULTURE

FARWA FAKHAR, RUKHAMA HAQ AND SHAGUFTA NAZ*

[1]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

*Corresponding author's email: mailto:drsnaz31@hotmail.com

Abstract

Viroid diseases are prevalent in all grapevine-cultivating regions around the world, affecting fruit quality and yield and posing a significant threat to global grapevine production. Meristem culture is a highly effective method for eliminating pathogens in *In vitro* propagated crops like grapevine. In this study, we present a protocol for producing GYSVd-2-free grapevine plantlets through apical meristem tip culture from indirect or direct organogenesis. Three different apical meristem sizes (0.5, 0.7, and 1.0 mm) were excised and cultured in Murashige and Skoog medium supplemented with different concentrations and combinations of cytokinin and auxin to determine the optimum size for viroid elimination. The RT-PCR analysis (indexing) was conducted on meristem- or callus-derived shoots of varying sizes (0.5, 0.7, and 1.0 mm). The study reveals that the 0.5 mm size was optimal for the GYSVd-2 elimination via indirect or direct organogenesis. The current research focused GYSVd-2 on detecting and eliminating in Pakistani grapevine cultivars to produce certified, healthy, viroid-free plantlets, with the potential to improve viroid-free plant production in the viticulture industry. Our investigation provides evidence that the efficiency of viroid elimination through apical meristem tip culture is an effective method for producing viroid-free grapevines in the future.

Key words: *Grapevine yellow speckle viroid -2*; Apical meristem tip culture; Reverse transcriptional Polymerase chain reaction; Plant growth hormones; Grapevine cultivars; Callus formation**;** Viroid elimination

Introduction

Grapevine is a highly valuable fruit crop from the *Vitaceae* family, one of the oldest and most widely cultivated perennial crops (Akram *et al*., 2020; Tang *et al*., 2024; Ren *et al*., 2025). The plant, originally from the Caucasus and western Asia, has since spread its significance to the American and European regions (Perrone *et al*., 2017). In Pakistan, the crop is listed among the top five produced fruit crops, and the country ranked 54th in the global production, with 78,034,332 tons (Fatchiyah *et al*., 2025). As an economically significant crop, it is utilized in various commercial products, such as juice, wine, seed oil, and raisins (Martin *et al*., 2020). In addition to being utilized as table fruit, grape waste, known as pomace, is also used as a raw ingredient in the cosmetic, medicinal products, and food industries (del Castillo, 2025; Wang, 2025).

Viroid diseases are prevalent in all grapevine-cultivating regions around the world, affecting fruit quality and yield, thereby limiting sustainable production and posing a significant threat to global grapevine production. (Moisseyev *et al*., 2025). Global reports have identified seven grapevine viroids: *grapevine yellow speckle viroid 1*, *grapevine yellow speckle viroid 2*, *hop stunt viroid*, *citrus exocortis viroid*, *Australian grapevine viroid*, *Japanese grapevine viroid*, and *grapevine latent viroid* (Morgan *et al*., 2023; Martelli, 2017).

Above all, *Grapevine yellow speckle viroid-2* is a significant latent viroid that severely impacts grapevine production (Fuchs *et al.*, 2025). GYSVd-2 is a viroid found only in grape cultivars and belongs to the Pospiviroidae family (genus Apscaviroid). GYSVd-2 is the smallest known pathogen, single-stranded, 360–365 nucleotides and noncoding (Moisseyev *et al.*, 2025). GYSVd-2 causes symptoms that are certain to specific environmental conditions, such as specific areas or years (Zhang *et al*., 2012; Di Serio *et al*., 2017). Symptoms are more evident when temperatures are high in the spring, above average, and leaves are exposed to direct sunlight. The prevalence of GYSVd-2 was low when the maximum mean temperature in mid-spring of another year was close to average (Habili, 2017). GYSVd-2 is a condition characterized by pinhead-sized yellow spots along the core and lateral veins of the grapevine leaf (Habili, 2017). The high variability in GYSVd-2 symptom expression may be attributed to seasonal variations in geography, cultivar, and temperature. The GYSVd-2 vector is not known, but natural transmission has been reported (Dahan *et al.*, 2024). Vegetative propagation is a method of disease transmission. There have been reports of spreading in vineyards by pruning instruments; however, this information could not be verified (Habili, 2017). GYSVd-2 is unknown for pollen transmission, and apscaviroids are not known to be transmitted through pollen (Hammond, 2017).

Management of grape pathogen diseases mainly focuses on detecting, preventing, and suppressing virus transmission (Alimzhanova *et al.*, 2025; Nanekar *et al.*, 2025). Sensitive pathogen detection and using healthy propagation material can prevent virus introduction into newly planted vineyards. The grapevine viroids primarily transmit through vegetative propagation (Buciumeanu *et al.*, 2024). Identifying grapevine viroids in vineyards is

Received: 13-05-2025 | Revised: 16-02-2026 | Accepted: 20-02-2026 | Online: 14-04-2026

crucial for facilitating effective control measures. Diagnostic methods have been developed to detect and quantify pathogens in vineyards and stock plantations to prevent the spread of viruses and viroids. Using reliable methods for grapevine pathogen detection is crucial for efficient management, is used in certification programs and field-based vine sanitation monitoring. PCR-based diagnostic methodologies have been used for pathogen diagnosis throughout the world owing to their sensitivity, accuracy, and reliability (Alashi *et al.*, 2025). RT-PCR has been found to be the most effective and accurate testing method for indexing.

The removal of viroids from crops propagated through vegetative methods is important. Meristem tip culture is one of the current method for eradicating viruses and viroids. Virus elimination therapy can be utilized in the production of viroid-free plants (Tang *et al*., 2024). Micro-propagation via the apical meristem tip culture technique first described by Morel (1941) for grapevine shoot tips, has been successfully applied to various plant species. Meristem culture is commonly used for eliminating plant pathogens due to its high growth potential, genetic stability, and cell division rate (Al Ghasheem, 2022; Rajesh *et al*., 2024). This method is a highly efficient method for rapidly multiplying large quantities of plant material. The size of the meristem portion is crucial for viroid eradication, as smaller portions have lower viroid concentrations. The smaller size facilitated precise excision and ensured a higher concentration of actively dividing cells, creating favorable conditions for efficient pathogen elimination. Tissue culture methodologies such as direct (organ) and indirect organogenesis (callus) have also been used for viroid elimination. Plant regeneration by direct and indirect organogenesis is a rare phenomenon in grapevine. The optimal media composition is crucial for enhancing the yield and quality of grapevine. Successful regeneration is influenced by the explant size, *In vitro* environmental conditions, and the combination of growth regulators in the medium. Several reports have explained regeneration in grapevine tissue culture by optimizing media components and explant type (Bouquet & Torregrosa, 2003; Gribaudo *et al.*, 2006; Maliogka *et al.*, 2009; Skiada *et al.*, 2013; Shatnawi *et al.*, 2011; Doroshenko *et al.*, 2021; Hu *et al.*, 2021; Miljanić *et al.*, 2022; Kim *et al.*, 2023; Bettoni *et al.*, 2024; Rasool & Naz, 2024).

This study focuses on evaluating the impact of meristem size and media compositions on (1) plant growth success in seven grapevine cultivars and (2) the production of viroid-free grapevine plants through direct and indirect organogenesis. Currently, there are no reports on the production of viroid-free grapevine cultivars through indirect organogenesis to the best of our knowledge. This study presents the first successful *In vitro* propagation protocol for the production of GYSVd-2-free grapevine plants in Pakistan. The results of current research will provide a technical platform for GYSVd-2-free grapevine plant production for certification.

Materials and Methods

The workflow of the current research study is illustrated in Fig. 1.

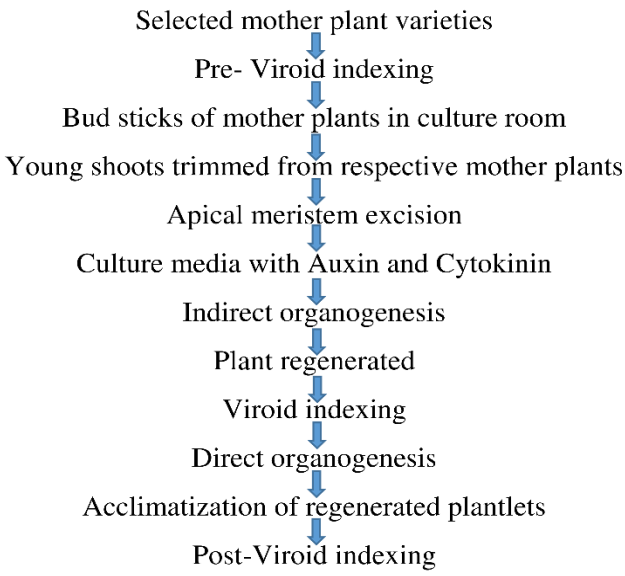

Fig. 1. Flowchart showing the apical meristem tip culture-based method to get rid of GYSVd-2 from *Vitis vinifera* cultivars.

Leaf sample collection and study area: The present study utilized seven grapevine (*Vitis vinifera*) cultivars: King Ruby (V1), Thomson seedless (V2), Flame seedless (V3), Crimson seedless (V4), Golden Italian Muscat (V5), Italian Michale Parali (V6), and Italian Pizzuluto (V7). The leaves surrounding the meristems were carefully removed were collected in 2022 during April and September from various districts of Quetta, the capital of Balochistan with the geographic location Pakistan-Gulistan Qilla Abdullah, SK Big Vineyard, Agha Sb. Pishin, and Viti Culture Centre Pishin. Samples were thoroughly cleaned, placed in resalable polythene bags, labeled with specific variety codes, then transported in a thermopore boxes containing ice, and transported overnight to preserve their integrity. Upon arrival, the samples were stored at -80°C prior to RNA extraction. All procedures were conducted in the Plant Biotechnology Laboratory, Pakistan.

Detection of *Grapevine Yellow Speckle Viroid-2* in mother plants by RT-PCR: The viroid infection status of respective mother plant cultivars was determined using the RT-PCR method (Mahmood *et al*., 2022). Total nucleic acid (RNA) was extracted from 0.8g of grapevine petiole and midrib in each sample using the CTAB (cetyltrimethylammonium bromide) method. Samples were pulverized, homogenized in CTAB buffer, incubated to induce cell lysis, and treated with chloroform for phase separation. The RNA-containing aqueous phase was isolated, precipitated with isopropanol, centrifuged, washed with ethanol, air-dried, and resuspended in RNase-free water for further analysis. Thermo Fisher Scientific Inc.'s Nanodrop ^{TM}-1000 at 260 and 280 nm was utilized to check the quantitative analysis of RNA. Furthermore, using a 1% agarose gel, RNA integrity was determined. RNA was preserved at -80ºC for further utilization. (Iandolino *et al*., 2004). The first-strand cDNA was synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, following the manufacturer's instructions. The cDNA synthesised (20 µl volume) included reactive components such as 3µl RNA template, 7µl RNase-free water, 1µl 100 µM random primer, 4µl 5X RT buffer, 0.5µl 40 U RNase inhibitor (RiboLockTM RNase inhibitor kit), 1.5µl 50 mM DDT, 2µl 10 mM dNTPs, and 1µl 200 U

Mu-MLV reverse transcriptase enzyme. The PCR tubes were incubated at 42ºC for 60 minutes. Then the DNase treatment process was utilized to eliminate extraneous DNA from an RNA sample. PCR was performed in a reaction volume of 25 µl containing 3 µl cDNA, 10.5 µl RNase water, 2 µl 10mM dNTP's, 2.5 µl 25mM MgCl2, 4 µl 10X taq buffer with $(NH4)^2 SO^4$, 1 µl viroid primer (10 mM), 1µl 5U/µl Taq DNA polymerase (Thermo Scientific Fisher). The RT-PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 62°C for 40 s, and 72°C for 40 s, and a final elongation step at 72°C for 5 min. The two-step end-point RT-PCR was completed in a thermal cycler (Primus-96). The primer set (GYSVd-2 F and GYSVd-2 R) underwent 35 cycles, with a final extension at 72°C. The specific primer set for GYSVd-2 analysis is listed in Table 1. The amplification products were electrophoresed on 1.0 % agarose gels in 1XTAE buffer and visualized under UV illumination with ethidium bromide (0.8 g/mL).

GYSVd-2 elimination

Plant material, surface sterilization of *Vitis vinifera* shoot tips, apical meristem excision, culture media and growth conditions: In January 2023, 12 cm-long bud sticks with multiple dormant buds of selected plant cultivars were obtained from infected mother plants and transported to a plant biotechnology laboratory, Department of Biotechnology, Lahore College for Women University Lahore, Pakistan. In a culture room with a temperature of 18-23 degrees Celsius, nutrient-free distilled water was maintained. After three weeks, buds bursted and shoots was sprouted (Fig. 2a A-D). This study utilized newly formed, three-week-old young shoots from infected grapevine cultivars as an explant source. Surface sterilization was a critical step to eliminate contaminants from the shoot tips and ensure successful *In vitro* -propagation. Four variants of disinfection were tested to determine the optimal protocol. The leaves surrounding the meristems were carefully removed. For 5 minutes, the apical portions (1 cm) were washed in a jar with 3 drops of liquid detergent to remove surface dust particles as well as fungal and bacterial (microbial) spores. The shoot tips were then sterilized in 70% ethanol for less than 60 seconds to remove debris and sanitize the surface. Shoot tips were immersed in a 10% sodium hypochlorite solution for different time durations to effectively eliminate microbial contaminants. The shoot tips were rinsed 3 times with autoclave H_2O to eliminate the remaining chemicals and detergent, which was done in a septic environment inside the laminar airflow cabinet. In this study, three replications of each variant were maintained.

After surface sterilization, three different apical meristem sizes (0.5, 0.7, and 1.0 mm) were excised by using a binocular dissecting microscope (Leica Wild M3Z) with 10–80X magnification under the aseptic conditions of a Laminar Air Flow hood. Fig. 2b shows the microscopic view of grapevine meristem. The culture medium was Murashige and Skoog (MS) basal medium, supplemented with 30 g L^{-1} sucrose, 0.1 g L^{-1}, myoinositol, and 8 g L^{-1} agar. The MS medium was supplemented with varying concentrations of PGRs (cytikinin and auxin) as per experimental requirement. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 30 minutes. All *In vitro* cultures were maintained in a room with a temperature of 23 ± 2°C, 56-60% humidity, and 16 hours of fluorescent light.

Effect of different concentrations of BAP on shoot induction: In this experiment, the respective meristem sizes were initially inoculated on MS medium supplemented with different concentrations of BAP (0.0, 0.5, 0.7, and 1.0 mg L^{-1}) to determine the optimal concentration for the induction of *Vitis vinifera* shoot (Fig. 3). The MS medium (basal) without BAP was used as a control.

Effect of 1.0 mg L^{-1} of BAP in combination with varying concentration of NAA and IAA on explant regeneration: Then, the microshoots derived from the respective sizes of meristems were transferred for further shoot elongation and root induction on MS medium supplemented with BAP (0.0, 1.0, and 1.0 mg L^{-1}) along with NAA (0.0, 0.1, and 0.2 mg L^{-1}) and IAA (0.0, 0.1, and 0.2 mg L^{-1}).

Detection of meristem or callus-derived shoots for GYSVd-2: After that, up to 160 callus or meristem-derived shoots of varying sizes (0.5, 0.7, and 1.0 mm) from the *In vitro* apical meristem tip culture method were randomly selected and indexed using RT-PCR to assess the impact of apical meristem size on the frequency of production of viroid-free grapevine. The RNA from *In vitro* -cultured grapevines of various cultivars was extracted, cDNA synthesized, and RT-PCR analysis was performed using viroid-specific primers. All the procedures were executed in the same way as described above in detection of *Grapevine Yellow Speckle Viroid-2* in mother plants by RT-PCR section.

Effect of 1.0 mg L^{-1} of BAP in combination with varying concentrations of NAA on root induction: Following confirmation of viroid-free status, actively growing, well-developed shoots derived from meristem or callus were shifted to root induction medium. To determine the optimal concentration of NAA, well developed shoots were subcultured in MS medium containing optimal BAP along with varying concentrations of NAA (0.1, 0.2, and 0.3 mg L^{-1}).

Acclimatization of GYSVd-2-free *Vitis vinifera* plantlets: The *In vitro* -established plantlets were thoroughly washed with double-distilled water to remove culture medium attached to the root. *Vitis vinifera In vitro* -established plantlets were then carefully transplanted from a culture jar to a plastic pot containing compost for further acclimatization. Compost significantly enhances the root development, expansion, and survival of acclimatized plants due to its rich nutrient content. The compost components included rice husk ash (50%), sugarcane mud (15%), coco peat (25%), humic acid (5%), and fungicides (Antracol® 70 WP and Dragon 700WG) (5%), respectively. The plastic pots, placed in a magenta box, were maintained in a plant incubator, watered, and covered. The box cover was removed for 30 minutes, then extended to 50 minutes daily. All the plantlets were maintained in plant incubator at 21-23ºC, white fluorescent lamps (40 µmol m^{-2} s^{-1}) with 16 h light duration. Then the plantlets were transferred to a sterile soil contained large pots and placed in greenhouse, where they continued to grow. The viroid-free plants survival rate was recorded.

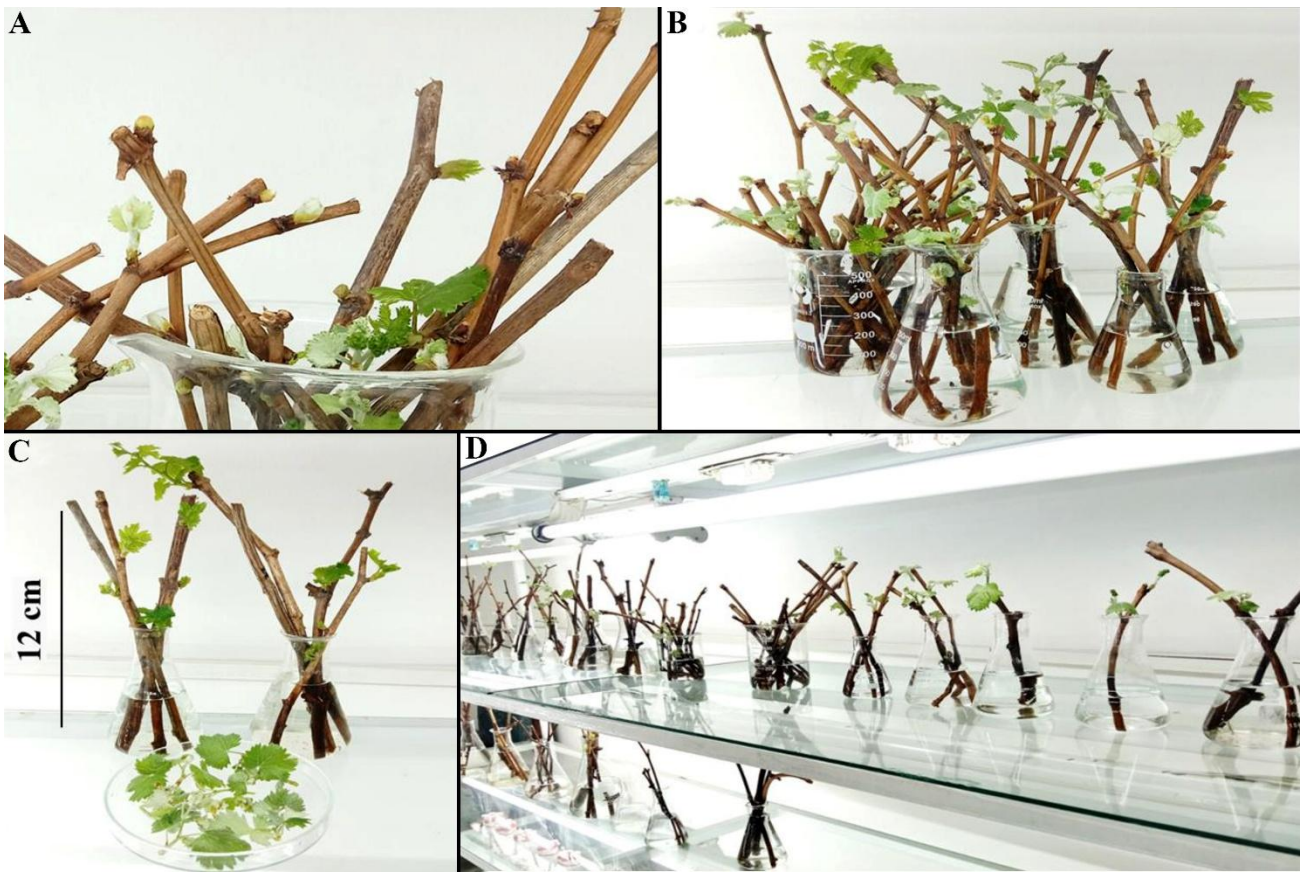

Fig. 2a. Flowchart showing the main steps of the shoot development protocol used to eliminate GYSVd-2 from infected cultivars. (A) Swelling, emergence, and elongation of shoots from bud sticks (B) Successful propagation of young shoots from bud sticks (C) Cutting shoot tips from the nodal part (D) A visual depiction of the shoot development process from *V. vinifera* bud sticks in the culture room.

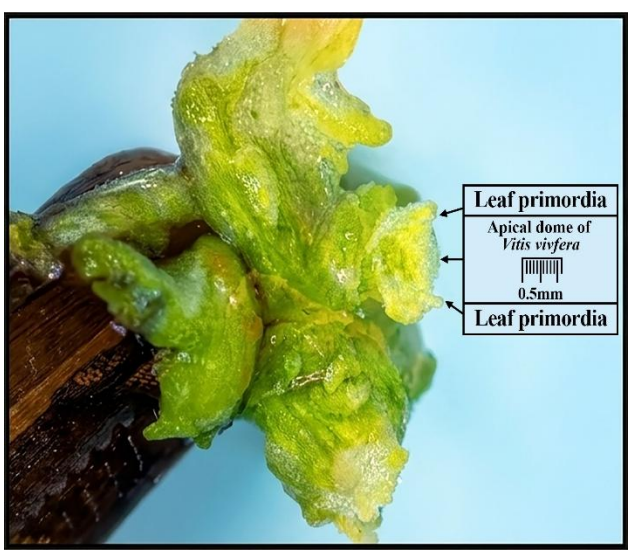

Fig. 2b. Microscopic view of grapevine meristem.

GYSVd-2 elimination detected by RT-PCR: Following the confirmation of the GYSVd-2-free status of grapevine plants produced through apical meristem tip culture, further analysis was carried out to ensure the accuracy of the results. Samples were taken at the acclimatized stage from newly developed leaves of micro-propagated plantlets. To confirm the amplification's proficiency, the positive control (already-known infected DNA sequences) for GYSVd-2 was also run.

Fig. 3. Three different sizes of meristematic *Vitis vinifera* inoculated on MS medium + BAP (1.0 mg L^{-1}).

Statistical analysis

The average value was employed to signify all data for each treatment. Analysis of variance (ANOVA) was used to determine differences between treatments. SPSS 22.0 software was used for all statistical analyses, and post hoc tests were performed using Duncan's multiple range test. Any p-value below 0.05 was considered statistically significant.

Results

Pre-viroid-detection of mother plant: Total RNA was isolated to check the occurrence of GYSVd-2 in Pakistani grapevine cultivars. Out of the seven samples tested, the RT-PCR results showed amplification in four samples (Thomson seedless, Golden Italian Muscat, Crimson seedless, and Italian pizzuluto), in which the positive control exhibited a 363-bp band, while no such band was observed in the negative and non-template controls. The amplification results are presented in Table 2 and Fig. 4A. No amplification was detected in King Ruby, Flame Seedless, and Italian Michele Parali. Consequently, GYSVd-2-infected cultivars were subjected to viroid elimination procedures.

Apical meristem tip culture: This study investigates the effect of different amounts and combinations of cytokinin and auxin, especially BAP, IAA, and NAA, on plant growth. Two distinct modes of regeneration were identified: indirect organogenesis (callus formation) and direct organogenesis (organ formation).

Effect of sodium hypochlorite (10%) and shoot tip immersion durations (min.) on contamination and survival rate (%): As presented in supplementary Information Table 3 and shown in Fig. 5, the sterilization of the 1 cm shoot tips exhibited significant differences depending on the time following the sterilization variant. In the first variant, the shoot tips were immersed in a 10% bleach solution for 5 minutes, and resulting in a high contamination rate of 73.32% was observed. The survival rate was 26.63%, indicating that only eight of the shoot tips were able to survive. The five-minute test showed that the sterilization process was very poor in eliminating contaminants after the first week. In the second variant, the shoot tips were immersed in the same 10% bleach solution but for a longer duration of 8 minutes. The contamination rate was 0.00%, indicating that none of the shoot tips were contaminated. The survival rate of shoot tips was 100.00% after one week. Similar results were found in the third variant. Immersion for 8–10 minutes was found to be optimal for achieving effective surface sterilization without causing damage, and it was chosen as the sterilization protocol for the remaining infected varieties. And this optimal protocol proved optimum for all remaining varieties as well. In the fourth variant, shoot tips were immersed in a 10% bleach solution for 15 minutes. As the immersing time increased, the contamination rate was 86.62%, while the survival rate was only 13.32%, indicating that only a small percentage of shoot tips survived the first week. However, 100.00% survival rate were noticed at 8–10 minutes immersion duration. The 15-minute period did not prove effective for further surface sterilization or regeneration purposes.

Table 1. The primer set utilized in the RT-PCR assay for detecting GYSVd-2 in *Vitis vinifera* spp.

Viroid genus	Oligonucleotides name	Sequence 5'-3'	Amplicon size (bp)	References
Apscaviroid	*GYSVd-2-F*	GACCTGCAGAGAAAAGAAGAAGG	363	Jiang *et al*., 2009; Zhang *et al*., 2012
	GYSVd-2-R	GCTCGACTAGCGGAGGC		

Table 2. *Grapevine Yellow Speckle Viroid* 2 status in Pakistani grapevine cultivars.

Pre viroid indexing by RT-PCR		Viroid name	Viroid indexing of callus or meristem-derived shoots			Post indexing of *In vitro* grown plantlets by RT-PCR	Viroid free plants at acclimatized stage
Cultivars Name	Cultivars Code or ID	GYSVd-2	Apical meristem size (mm)				Survival rate (%)
			0.5	0.7	1.0	0.5	
King Ruby	V1	-	-	-	-	-	100% (28/28)
Thomson seedless	V2	+	-	-	+	-	100% (30/30)
Flame seedless	V3	-	-	-	-	-	100% (35/35)
Crimson seedless	V4	+	-	+		-	100% (35/35)
Golden Italian Muscat	V5	+	-	-	+	-	100% (18/18)
Italian michale parali	V6	-	-	-	-	-	100% (21/21)
Italian pizzuluto	V7	+	-	-	-	-	100% (31/31)

Table 3. Effect of sodium hypochlorite (10%) and shoot tip immersion durations (min.) on contamination and survival rate (%).

Variant	Sodium hypochlorite (%)	Shoot tip immersion duration (min.)	No. of shoot tips	No. of contaminated explants (after 1 week)	Contamination rate (%)	Survival rate (%)	Sterilization effectiveness
1	10	5	30	22	73.32^{b}	26.63^{b}	Very poor
2	10	8	30	0	0.00^{c}	100.00^{a}	Optimal
3	10	10	30	0	0.00^{c}	100.00^{a}	Optimal
4	10	15	30	26	86.62^{a}	13.32^{c}	Not entirely effective

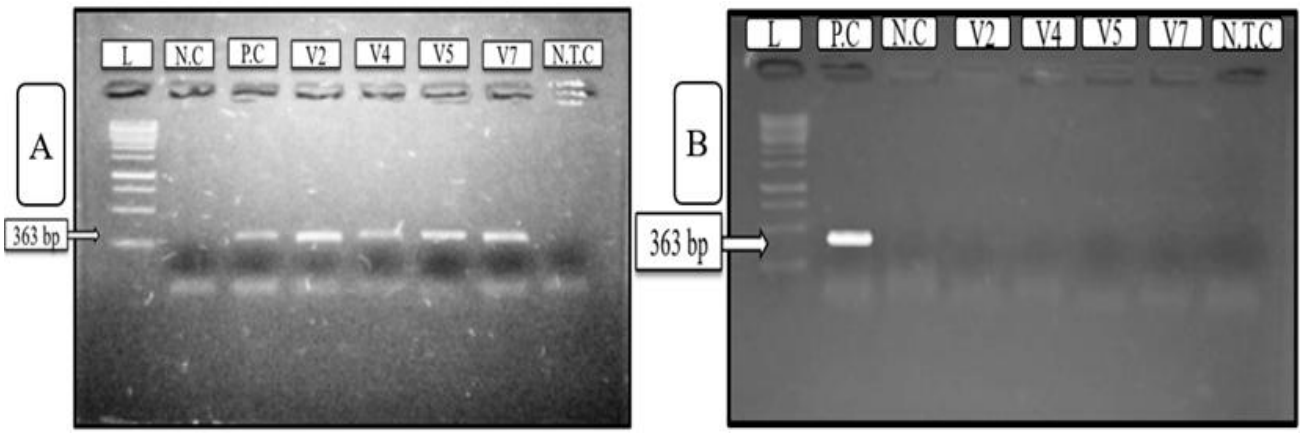

Fig. 4. PCR amplification of *Grapevine yellow speckle viroid-2* (A) Pre-indexing RT-PCR results from selected cultivars; L represents the 1 Kb GeneRuler ladder with negative, positive, and NTC control, Samples (V2, V4, V5, V7) showing amplification bands (B) Post-indexing RT-PCR results from selected cultivars; L represents the 1 Kb GeneRuler ladder with negative control, positive control, Samples (V2, V4, V5, V7) showing no amplification bands and NTC control.

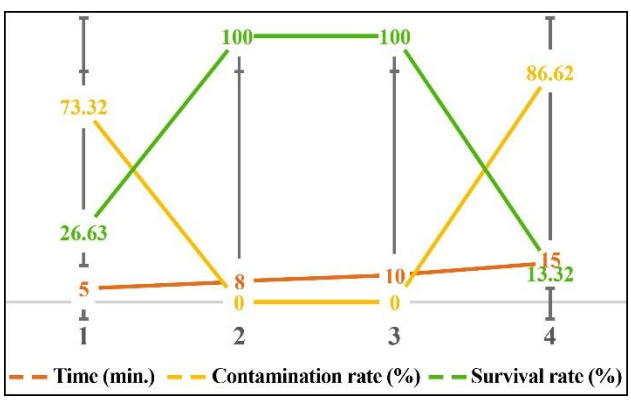

Fig. 5. The effect of 10% sodium hypochlorite on sterilization of shoot tip. Contamination and survival rate with different shoot tip immersion durations (5, 8, 10, and 15 min). Within each color, the mean value with the standard deviation was statistically significant based on Duncan's ≤0.05. Each value is the mean of three replicates.

Effect of different concentrations of BAP on shoot induction: The study observed the variations in duration of shoot induction (days), average number of shoots per explant, success rate of shoot induction (%), and shoot length (cm) varied among the various concentrations of BAP tested. All cultures were inoculated with MS medium formulations with BAP-induced shoots. It was observed that the addition of BAP led to shoot induction in a shorter period compared to the control (42.0 days). At an apical meristem size of 0.5 mm, the MS medium supplemented with 1.0 mg L−1 BAP resulted in the shortest shoot induction period of 22.0 days, followed by 0.7mg L−1 BAP with an induction period of 23.2 days, and 0.5 mg L−1 BAP, which induced shoots in 28.5 days. However, the differences were not statistically significant ($p>0.05$), as shown in Table 4.

The highest average number of shoots per meristem (2.0 shoots) was observed in MS medium with 1.0 mg L−1 of BAP, followed by 0.7 mg L−1 BAP (1.0 shoot) and 0.5 mg/L BAP (1.0 shoot), which demonstrated significant differences compared to the control treatment (1.0 shoot) (Table 4). The highest shoot induction success rate (90.2±0.3%) was observed at 1.0 mg L^{-1} BAP concentration, compared to 0.7 mg L^{-1} (88.1±0.2%) and 0.5 mg L^{-1} (85.3±0.5%). Nonetheless, the differences were not statistically significant ($p>0.05$), as shown in Table 4. The highest mean length of shoot (1.2±0.1 cm) was observed at 1.0 mg L^{-1} of BAP, then at 0.7 mg L^{-1} (0.4±0.5 cm) and 0.5 mg L^{-1} (0.2±0.5 cm). These concentrations exhibited significant differences compared to the control treatment (0.1±0.5 cm) (Table 4). At 0.7 mm apical meristem size, it was found that different BAP concentrations led to the significant difference in shoot induction. Regarding the effect of different concentrations of BAP applied to the grapevine cultivars, shoot induction was observed in all BAP concentrations. However, the minimum days of shoot induction (23), highest mean number of shoots (2.03±0.0), highest success rate of shoot induction (90.1±0.1%), and mean shoot length (0.9±0.1 cm) were recorded in 1.0 mg L^{-1} of BAP.

At 1.0 mm apical meristem size, the mean values recorded ranged from 22 to 28.0 days, with 1.0±0.0-2.0±0.0 shoots, an induction rate of 88.0±0.2% to 90.1±0.1%, and a length of 0.3±0.0 to 0.9±0.3%. It was observed that the addition of 1.0 mg L^{-1} of BAP led to shoot induction in a shorter period, maximum shoot number, highest shoot induction success rate, and highest length (Table 4). Fig. 6 shows the meristematic cell proliferation and micro-shoot formation on MS culture medium supplemented with 1.0 mg L^{-1} of BAP. BAP at 1.0 mg L^{-1} was found to be optimum when used individually. Thus, in the present study, 1.0 mg L^{-1} of BAP was used in culture medium for shoot elongation and proliferation.

Effect of BAP, NAA, and IAA on explant regeneration: After successful shoot induction, approximately five to six weeks of culture, the micro-shoots were sub-cultured on MS medium with different concentrations and combinations of plant growth regulators (BAP, NAA, and IAA) (1.0+0.1+0.1) for shoot and root induction. Callus induction occurred at the base of the micro-shoots except in the basal media. The light yellowish or white to light green color of the callus was observed to indicate active

cell division. The growth of callus continued until the 8th week with the presence of shoots, followed by root formation in the 9th week. There was visible evidence that mass of undifferentiated cell tissues occurred in the presence of the combination of high auxin and cytokinin concentrations. These excess concentrations led to uncontrolled cell division, proliferation of callus tissue, and formation of large, undifferentiated callus masses, triggering shoot root formation and subsequent organogenesis. The success rate (frequency) of callus induction was 97%. Indirect organogenesis with healthy plantlets cultured on MS medium supplemented with (BAP, NAA, and IAA) (1.0+0.1+0.1 mg L^{-1}), was successfully induced from different cultivars of grapevine Fig. 7a(A-C). Calluses from all cultures showed contamination in the MS medium containing 1.0+0.2+0.2 mg L^{-1} of BAP, NAA, and IAA, but shoot and root development continued (Fig. 7b).

Viroid indexing of callus or meristem-derived shoots: RT-PCR indexing of each size (0.5, 0.7, and 1.0 mm) was performed after callus or meristem-derived shoot multiplications. The presence of GYSVd-2 in infected cultivars varied across the three sizes. RT-PCR results for GYSVd-2 are present in Table 2 and displayed in Fig. 8 across three different meristem sizes (A-0.5, B-0.7, and C-1.0 mm), indicating the presence of the viroid in callus or meristem-derived shoots. RT-PCR results confirmed that all samples from established cultures of 0.5 mm were negative, indicating that 0.5 mm is optimal for viroid-free grapevine production in selected cultivars. RT-PCR showed that 0.7 mm established grapevine cultures from meristem or callus were moderately suitable, potentially containing one positive sample, and 91% of propagated cultivars were viroid-free. The apical meristem size of 1.0 mm was deemed less suitable for producing viroid-free grapevines due to positive results obtained from RT-PCR. Thus, the investigation revealed that 0.5 mm explant size produced 100% viroid-free plants compared to 0.7 and 1.0 mm (91%, 88%).

Effect of 1.0 mg L^{-1} of BAP in combination with varying concentration of NAA on root induction: *In vitro* raised elongated shoots after indexing were shifted to MS medium with 1.0 mg L^{-1} of BAP along various concentration of NAA ranging from 0.1- 0.3. The optimal shoot induction response (16 days) and maximum number of roots per explant were observed with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA in MS medium, resulting in a 96% success rate and the highest root length of 3.9 cm (Table 5). In the current study, the newly produced shoots (meristem-derived shoots) were successfully induced through direct organogenesis without the intermediation of callus. No callus was observed from 1.0 mg L^{-1} of BAP with different concentrations of NAA treatments. After 6 weeks of culture, the treatment of 1.0 mg L^{-1} of BAP with 0.1 mg L^{-1} of NAA was found to be the most optimal for direct organogenesis.

The highest number of roots, with a mean of 2.1 roots per explant, was obtained from 1.0 mg L^{-1} of BAP with 0.1 mg L^{-1} NAA in MS medium. The medium with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA was found optimum for success rate of shoot induction, number of root per explant, and shoot length. Direct organogenesis with healthy plantlets is shown in Fig. 9(A-C). Comparing among the phytohormones, using them alone or combination with each other, combination of BAP+ NAA was found to be the best for direct organogenesis.

Transplantation: All *In vitro* -established viroid-free *Vitis vinifera* plantlets via indirect or direct organogenesis were easily transplanted (Fig. 10A). Fig. 10B and C displays healthy grapevine cultivars that successfully acclimatized in a greenhouse environment and survived outdoors. The plant survival rate remained consistent (100%) in both the greenhouse and growth chamber (Table 2).

Table 4. Effect of different concentrations of BAP (mg L^{-1}) on the induction of *Vitis vinifera* shoot.

Meristem size (mm)	MS media + Con. of BAP (mg/L)	Duration of shoot ınduction (days)	Average no. of shoots formed per meristem	Success rate of shoot induction (%)	Measurement of shoot length recorded after 2 months (cm)
Control	0.0	42.0 ± 0.2^{a}	1.0 ± 0.0^{b}	75.0 ± 0.5^{j}	0.1 ± 0.5^{j}
0.5	0.5	28.5 ± 0.4^{b}	1.0 ± 0.0^{b}	85.3 ± 0.5^{i}	0.2 ± 0.5^{h}
	0.7	23.2 ± 0.3^{h}	1.0 ± 0.0^{h}	88.1 ± 0.2^{e}	0.4 ± 0.5^{e}
	1.0	22.0 ± 0.1^{j}	2.0 ± 0.0^{a}	90.2 ± 0.3^{a}	1.2 ± 0.1^{a}
0.7	0.5	28.2 ± 0.2^{c}	1.0 ± 0.0^{b}	86.6 ± 0.5^{h}	0.2 ± 0.1^{i}
	0.7	25.2 ± 0.2^{e}	1.0 ± 0.0^{b}	88.1 ± 0.1^{f}	0.3 ± 0.1^{g}
	1.0	23.3 ± 0.3^{g}	2.0 ± 0.0^{a}	90.1 ± 0.1^{c}	0.9 ± 0.1^{b}
1	0.5	28.1 ± 0.1^{d}	1.0 ± 0.0^{b}	88.1 ± 0.2^{d}	0.3 ± 0.0^{f}
	0.7	24.2 ± 0.3^{f}	1.0 ± 0.0^{b}	88.0 ± 0.5^{g}	0.5 ± 0.0^{d}
	1.0	22.2 ± 0.2^{i}	2.0 ± 0.0^{a}	90.1 ± 0.1^{b}	0.9 ± 0.3^{c}

Table 5. Effect of BAP and NAA concentrations in MS medium for *In vitro* root induction.

MS media+ BAP (mg/L)	MS media+ NAA (mg/L)	Duration of root Induction from shoots (days`)	No. of roots formed per explant	Success rate of root ınduction (%)	Measurement of root length (cm)
0.0	0.0	28.03 ± 0.5^{a}	2.0 ± 0.5^{d}	75.09 ± 0.1^{d}	1.24 ± 0.1^{d}
1.0	0.1	16.10 ± 0.1^{d}	6.1 ± 0.1^{a}	96.16 ± 0.1^{a}	3.91 ± 0.0^{a}
1.0	0.2	19.13 ± 0.1^{b}	4.1 ± 0.1^{b}	82.13 ± 0.1^{c}	3.07 ± 0.0^{b}
1.0	0.3	18.01 ± 0.1^{c}	3.0 ± 0.0^{c}	88.16 ± 0.1^{b}	2.80 ± 0.0^{c}

Data represent mean of three repeats. Values represent the mean ± standard error (SE). Within the same column, values followed by the same letter are not significantly different ($p>0.05$)

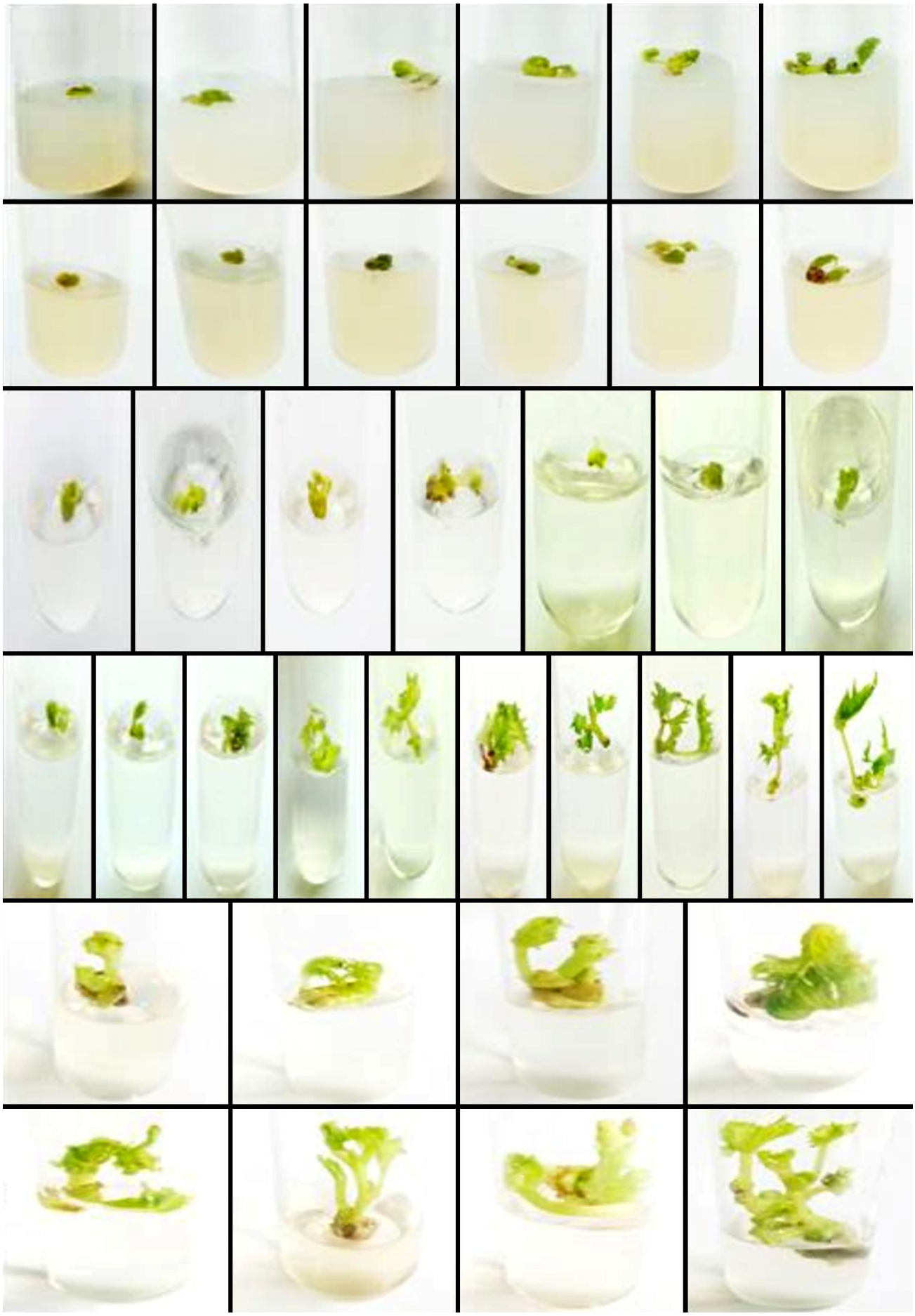

Fig. 6. Meristematic cell proliferation and micro-shoot formation on MS culture medium supplemented with 1.0 mg L^{-1} of BAP.

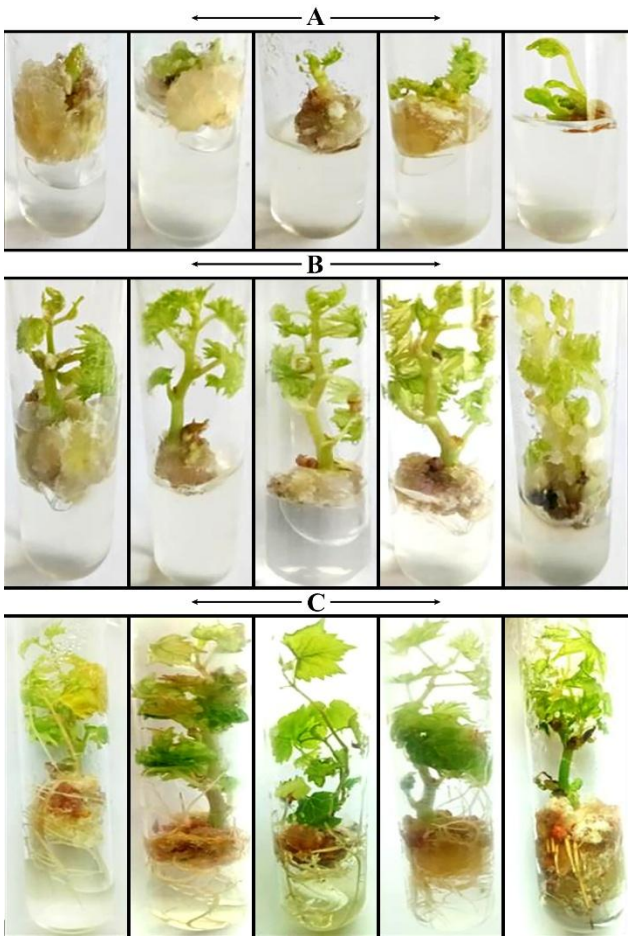

Fig. 7a. Indirect organogenesis with healthy plantlets Cultured on MS medium supplemented with (BAP, NAA, and IAA) (1.0+0.1+0.1) (A) Induction of Callus at the base of the micro-shoots (B) Formation of multiple shoots from the callus (C) Rooted shoot.

Fig. 7b. Indirect organogenesis with contaminated plantlets Cultured on MS medium supplemented with 1.0+0.2+0.2 mg L^{-1} of BAP, NAA, and IAA.

Post-Viroid indexing of Acclimatized plantlets: As presented in Table 2 and shown in Fig. 4B, negative results were detected in 0.5 mm *In vitro* -grown plantlets produced from both indirect and direct organogenesis. The respective infected cultivars were not infected with GYSVd-2, and no bands were observed in all plant, non-template, and negative controls. Thus, the investigation revealed that the plants grown through a 0.5 mm apical meristem (indirect and direct organogenesis) were found to be 100% viroid-free and healthy.

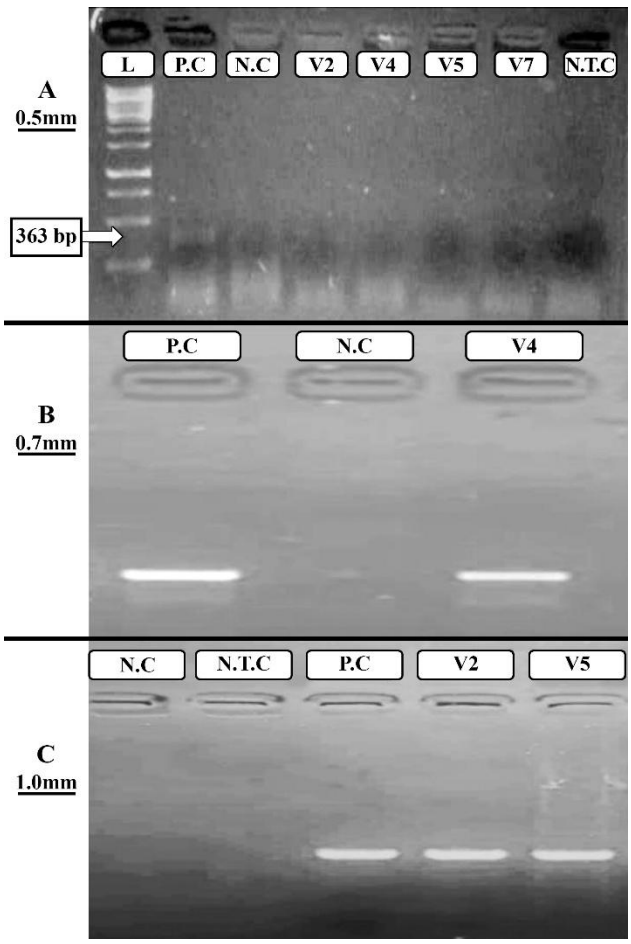

Fig. 8. RT-PCR results of callus or meristem-derived shoots (A) 0.5mm apical meristem size; Left to right: 1kb ladder, positive control, negative control, samples (V2, V4, V5, V7) showing negative GYSVd-2 and NTC (non-template control i.e. nuclease free water) B) 0.7mm apical meristem size; Left to right: positive control, negative control and positive sample (V4) (C) 1.0 mm apical meristem size; Left to right: negative control, NTC, positive control, and positive samples (V2, V5).

Discussion

Viroid infection significantly impacts grapevine yield and quality, necessitating the need for effective management of viroid-free plant material to ensure the sustainability of grapevine production. In this study, we successfully developed a reliable *In vitro* apical meristem tip culture protocol that eliminate GYSVd-2 from Pakistani grapevine cultivars. The findings provide critical insights into the impact of concentrations and combinations of phytohormones (cytokinins and auxins) on plant growth success and meristem size in producing viroid free grapevines. The study also offers valuable insights into the rapid multiplication of a perennial plant, ensuring high survival rates in regenerated plantlets and effectively eliminating GYSVd-2. However, limited evidence is reported regarding grapevine viroid elimination in Pakistan.

Our pre indexing results revealed that four selected grapevine cultivars were infected with GYSVd-2. Apical meristem tip culture was employed as an effective strategy to produce GYSVd-2-free plants. Following apical meristem tip culture technique, indexing viroid detection revealed a remarkable frequency of production of viroid-free grapevine (100%) from 0.5mm apical meristem. Microorganism contamination in tissue culture failure is primarily due to the surrounding environment. Microbial contamination in *In vitro*

culture can lead to uneven growth, tissue necrosis, and reduced shoot proliferation, negatively impacting plant growth. Decontamination methods like surface sterilizing agents and washing in running water are effective but require standardized concentration, immersion duration, and sequencing to ensure viability and minimize shoot tip injury.

The study conducted trials to determine the most effective sterilization durations for *Vitis vinifera* shoot tips. The explants showed optimal results after a 5-minute liquid detergent and tap water treatment, a less than 60 seconds 70% ethanol treatment, an 8-10 minute 10% sodium hypochlorite solution treatment, and three times rinsing with autoclave water. After a week of culture, the eight- and ten-minute immersion durations resulted in 100% survival and 0% contamination. Five- and fifteen-minute shoot tip immersion periods in 10% sodium hypochlorite did not prove to be effective due to high contamination and low survival rates. Our surface sterilizing results agree with Bashi and Toma (2024) in their findings that low sodium hypochlorite concentrations increase plant contamination, while high concentrations cause plant death.

Apical meristem is widely employed *In vitro* across various species to induce direct and indirect regeneration utilized in micropropagation strategies. Ilyas *et al*., (2019) successfully established an *In vitro* microshoot tip culture protocol using two grapevine cultivars. Furthermore, apical meristem has been widely utilized to eliminate pathogens in diverse plant species like Humulus lupulus (hops), sugarcane, and sweet potato varieties (Grudzinska *et al*., 2006; Krishnamurthy & Bahadur, 2015; Adhikary *et al*., 2021; and Wondimu *et al*., 2012). Several studies have documented the successful generation of pathogen-free grapevine via apical meristem tip culture. Hemmati & Dorani, 2023 highlight that meristem culture is a widely used and effective method for removing viral pathogens from infected plants *In vitro*. El Sayed *et al*., (2023) also reported that meristem and shoot-tip culture are commonly used for producing disease-free plants in the production of viroid-free stock.

Different authors have reported on the impact of the mineral composition of the culture medium on the *In vitro* culture of grapevine (Skiada *et al*., 2013; Dev *et al*., 2019; Mukherjee *et al*., 2010; and Eftekhari *et al*., 2012). The combination of BAP, NAA, and IAA (1.0+0.1+0.1 mg L^{-1}) in MS medium led to indirect organogenesis, while direct organogenesis was obtained with concentrations of 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA. The study found that micro-shoots exhibited callus formation when excessive concentrations of BAP, NAA, and IAA were added. This study used a BAP concentration similar to Rasool and Naz (2024), which is essential for increasing multiplication in micropropagation procedures. San Pedro *et al*., (2017) stated that the dose of BAP was found to be adequate in promoting new buds with good development in other species.

Shoot tips are commonly used as explants in *In vitro* culture methods to eliminate pathogens from plant species due to their inherent pathogen-free status and strong meristematic growth potential. Apical meristem culture is a highly successful method in producing pathogen-free plants. The production frequency of viroid-free grapevines was significantly influenced by the apical meristem sizes (0.5, 0.7, and 1.0 mm). During the *In vitro* phase, the respective sizes of each variety continued to test for GYSVd-2 status. The obtained RT-PCR results showed that 0.5 mm meristem or callus-derived shoots eliminated 100% GYSVd-2 from infected cultivars, while 0.7-1.0 mm explants showed 91% and 88% elimination, respectively. The study found that 0.5 mm meristem culture was more effective in eliminating GYSVd-2 than 0.7 and 1.0 mm, confirming the absence of viroid in meristem- or callus-derived plants. Negative results demonstrated that the viroid was absent in 0.5 mm of meristem or callus-derived explants, but post-indexing was done at the final stage (acclimatization) to confirm its complete elimination. However, the survival rate (100%) was observed from the *In vitro* phase and acclimatized phase. Previous research on *Vitis* species indicates that smaller explants are more virus-free due to the absence of vascular tissues, where viruses typically reside. Research by Rasool *et al*., (2023), Shoubra (2022), and Fayek *et al*., (2009) reported that 0.5 cm long meristem tips are most effective for obtaining virus-free explants, which is in accordance with our findings.

The size of the meristem in meristem tip culture impacts survival and the frequency of producing viroid-free explants. Smaller meristems increase virus or viroid elimination probability, while survival depends on meristem size (Wang & Jiao, 2018; Karimpour *et al*., 2020; Karimpour *et al*., 2025). The study indicates that the success of vine viroid elimination is largely dependent on the efficiency of small meristems in eliminating GYSVd-2. This technique serves as a promising and reliable method for pathogen-free propagation and can also be implemented in viticulture to enhance plant health, quality, and productivity. The application of apical meristem tip culture minimizes economic losses from latent infections, boosts vineyard productivity, and offers a high success rate in obtaining viroid-free plants (Perez-Caselles *et al*., 2025). Apical meristem tip culture is crucial for producing pathogen-free stock plants. Prior research has demonstrated that apical meristem culture is effective in producing grapevine plants devoid of pathogens. The integration of certified clean plant material and apical meristem culture through micro-propagation represents a comprehensive strategy for the effective elimination of grapevine viroids. Despite advancements in apical shot tip culture, the complete eradication of certain pathogens remains a significant challenge. New sequence variations reveal molecular diversity and GYSVd-2 evolution, providing insights into host-viroid interactions that can guide the development of viroid resistance. The research suggests that combining apical meristem culture with chemotherapy, cryotherapy, and thermotherapy could potentially improve the efficacy of pathogen elimination in 0.7 and 1.0 mm size. The combination of effective treatments with meristem tip culture effectively eradicates pathogens in grapevine cultivars, broadening its applicability across various grapevine cultivars (Markovic *et al*., 2013; Markovic *et al*., 2015; Bettoni *et al*., 2019; Bettoni *et al*., 2021; Bettoni *et al*., 2022). Furthermore, specialized skill training is crucial for the successful implementation of these techniques. Apical meristem can significantly improve the sustainability, resilience, and adaptability of grapevine cultivation by overcoming limitations in modern horticulture. Prior researches did not address the viroid detection, elimination, and micropropagation techniques within a single framework in Pakistan. However, this study is the first to use meristem culture to produce GYSVd-2-free grapevine varieties in Pakistan and the first to investigate GYSVd-2 eradication from these cultivars. The novelty of this work lies in its integrated approach—combining viroid detection, elimination, and reproducible protocol into a single efficient system—establishing a new and effective method for producing viroid-free grapevine plantslets- a contribution not previously reported in the Pakistan scientific literature.

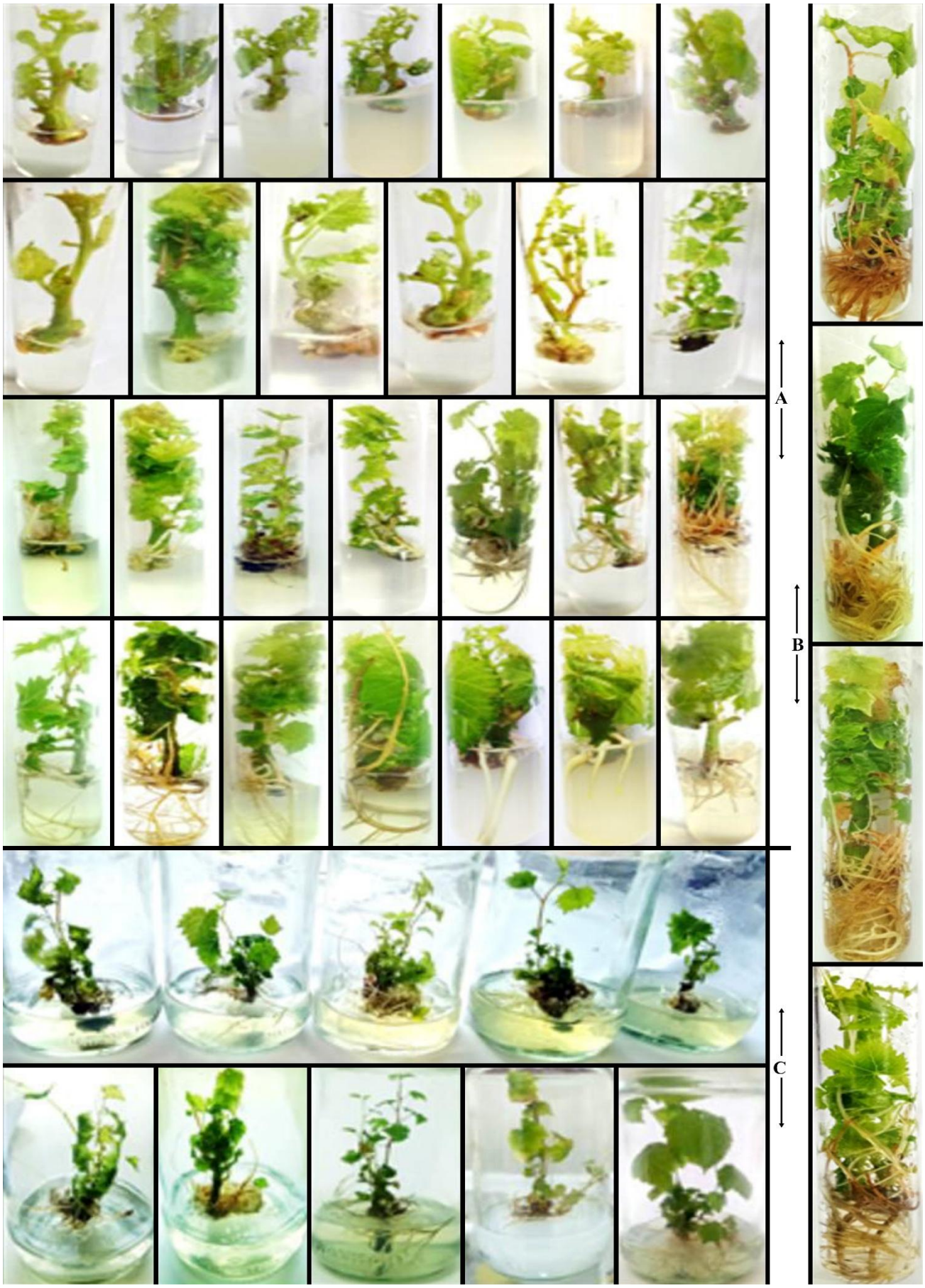

Fig. 9. Direct organogenesis with healthy plantlets Cultured on MS medium supplemented at 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} NAA concentrations (A) Proliferation of multiple shoots and Root development from shoot bases (B) Root Elongation and Plantlet Formation (C) Subculture for further proliferation and multiplication.

Fig. 10. Hardened *In vitro* established viroid-free *Vitis vinifera* plantlets (A) in Compost-Containing Pots (B) Acclimatization in growth chamber (C) Acclimatization in green house

Conclusion

RT-PCR is a highly specific and sensitive method for detecting viroid infection in grapevine cultivars. Micropropagation utilizing apical meristem culture is a promising technique for viroid eradication in grapevines. In the current study, we have standardized the direct and indirect organogenesis protocols for GYSVd-2-free grapevine cultivars production through apical meristem tip culture. The type of phytohormone (cytokinin and auxin) concentrations significantly affected the shoot and root induction. Viroid free or healthy grapevine plantlets could be produced by both indirect and direct organogenesis. Callus formation was observed on excess concentrations of BAP, NAA, and IAA. Direct organogenesis was observed on concentration of BAP and NAA. The viroid-free grapevine plants were acclimatized in the plant growth chamber and in the greenhouse, achieving a 100% survival rate. High survival rates, low contamination rates, and good growth performance of the plantlets across all cultivars highlight the potential of this technique for certification.

The current research successfully established an efficient micropropagation protocol for seven grapevine cultivars, achieving up to 100% GYSVd-2-free cultivars through 0.5 mm meristem tip culture. This investigation represents the first comprehensive data from Pakistan to simultaneously detect and eliminate GYSVd-2. The integration of viroid detection, elimination, and meristem tip culture technique in a single framework is a significant advancement in grapevine biotechnology. The outcomes not only contribute valuable baseline data on GYSVd-2 prevalence and disease aetiology in Pakistan but also provide a practical foundation for producing healthy, viroid-free plantlets, which can enhance vineyard productivity and support sustainable viticulture in Pakistan. In conclusion, apical meristem tip culture is a key technique in modern plant biotechnology for pathogen-free production. *In vitro* propagation and viroid-elimination protocols, we expect to significantly improve grapevine production in Pakistan, where GYSVd-2 is an important constraint. The study provides technical support for sustainable grapevine varieties and viticulture industry development.

Conflict of Interest: On the behalf of all authors**,** the corresponding author states that there is no conflict of interest.

Author's contribution: Farwa Fakhar: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Shagufta Naz:** Writing – review & editing, Supervision. All authors read and approved the final manuscript.

References

Adhikary, D., M. Kulkarni, A. El-Mezawy, S. Mobini, M. Elhiti, R. Gjuric and P. Bhowmik. 2021. Medical cannabis and industrial hemp tissue culture: present status and future potential. *Front. Plant Sci*., 12: 627240.

Akram, M.T., R.W.K. Qadri, M.J. Jaskani and F.S. Awan. 2020. Phenological and physicochemical evaluation of table grapes germplasm growing under arid subtropical climate of Pakistan. *Pak. J. Bot*., 52(3): 1011-1018.

Al Ghasheem, N. 2022. Effect of explants and auxins concentration on callus induction on peach (*Prunus persica* L. Batsch) micropropagation. *Texas J. Agri. Biol. Sci.*, 8: 115-121.

Alashi, R., S. A. M. Hosseini, Rastgou and V. Roumi. 2025. Detection of phytoplasmas associated with grapevine in Northwestern Iran. *Appl. Fruit Sci.*, 67(4): 203.

Alimzhanova, M., N. Meirbekov, Y. Syrgabek, R. Lopez-Serna and S. Yegemova. 2025. Plant-and microbial-based organic disease management for grapevines: A review. *Agri*., *15*(9): 963.

Bashi, A.Z.K. and R.S. Toma. 2024. *In vitro* Propagation of Two Grapes, *Vitis vinifera* L. cvs. superior and red globe: *vitis Vinifera* L.. *NTU J. Agric. Vet. Sci*., 4(2): 99-107.

Bettoni, J.C., G. Fazio, L. Carvalho Costa, O.P. Hurtado-Gonzales, M.A. Rwahnih, A. Nedrow and G.M. Volk. 2022. Thermotherapy followed by shoot tip cryotherapy eradicates latent viruses and apple hammerhead viroid from *In vitro* apple rootstocks. *Plants*, 11(5): 582.

Bettoni, J.C., M.R. Wang, J.W. Li, X. Fan, G. Fazio, O.P. Hurtado-Gonzales and Q.C. Wang. 2024. Application of biotechniques for In vitro virus and viroid elimination in pome fruit crops. *Phytopathol.,* 114(5): 930-954.

Bettoni, J.C., R. Bonnart, A. Shepherd, A.A. Kretzschmar and G.M. Volk. 2019. Cryopreservation of grapevine (*Vitis* spp.) shoot tips from growth chamber-sourced plants and histological observations. *Vitis*, 58(2).

Bettoni, J.C., Z. Markovic, W. Bi, G.M. Volk, T. Matsumoto and Q.C. Wang. 2021. Grapevine shoot tip cryopreservation and cryotherapy: Secure storage of disease-free plants. *Plants,* 10*:* 2190.

Bouquet, A. and L. Torregrosa. 2003. *Micropropagation of the grapevine (Vitis* spp*.)*. In *Micropropagation of woody trees and fruits* (pp. 319-352). Dordrecht: Springer Netherlands.

Buciumeanu, E.C., L.C. Guta and D.E. Vizitiu. 2024. Management of grapevine viral diseases. *Sci. Stud. & Res. Series Biol. / Studii Si Cercetari Stiintifice. Seria Biologie*, 33(1): 39.

Dahan, J., G.E. Orellana, J. Lee and A.V. Karasev. 2024. Occurrence of *grapevine yellow speckle viroid 2* and *Australian grapevine viroid* in Idaho grapevines. *Plant Dis.*, 108(4): 1121.

del Castillo, M.L.R. 2025. Table grapes: Health implications and approaches to improve the quality during the cultivation. *Adv. Bot. Res.*, 113: 179-210. Academic Press.

Dev, R., S. Singh, V. Dayal, K. Kumar and T.J.I. Singh. 2019. Standardization of *In vitro* hardening strategies for tissue cultured wine grape (*Vitis vinifera* L.) genotypes. *Int. J. Curr. Microbiol. App. Sci.*, 8(2): 2108-2117.

Di Serio, F., E.M. Torchetti, R. Flores and T. Sano. 2017. Other apscaviroids infecting pome fruit trees. In: *Viroids and satellites* (pp. 229-241).

Doroshenko, N., V. Puzirnov and L. Troshin. 2021. Optimization of grapevine clonal micropropagation. IOP Conference Series: Earth and Environmental Science, IOP Publishing. 022109.

Eftekhari, M., M. Alizadehand and P. Ebrahimi. 2012. Evaluation of the total phenolics and quercetin content of foliage in mycorrhizal grape (*Vitis vinifera* L.) varieties and effect of postharvest drying on quercetin yield. *Ind. Crop. Prod.*, 38*:* 160-165.

El Sayed, T.A., K.E. Saker, A.E. El Shorbagy, A.M. Mandour, M.J. Alajouz and A.M. Soliman. 2023. Characterization and elimination of grapevine fanleaf virus using thermotherapy in combination with meristem tip culture Egypt. *Acad. J. Biol. Sci. C, Physiol. and Molecul. Biol.*, 15(2): 101-117.

Fatchiyah, F., A.H. Naufal, C.V Alvionita and E. Suyanto. 2025. Virtual tracing of DFR mRNA and protein variabilities trace the ancestral origin of *Vitis vinifera* grapevine cultivars across geographic regions. *J. of Tropical Biodiv. and Biotech*., 10(3): jtbb16398.

Fayek, M.A., A.H. Jomaa and A.B.A. Shalaby. 2009. Meristem tip culture for *In vitro* eradication of *grapevine leaf roll-associated virus-1* (GLRaV-1) and *grapevine fan leaf virus* (GFLV) from infected flame seedless grapevine plantlets. *Iniciación a la Investigacion*, (4): 303.

Fuchs, M., M.A. Rwahnih, A.G. Blouin, J. Burger, K.M. Chooi, F. Constable and E. Varallyay. 2025. A list of eclectic viruses, virus-like diseases and viroids of grapevines that should not be considered for regulatory oversight: A global plea from virologists. *J. Plant Pathol.*, 1-12.

Gribaudo, G., J. Bondaz and I. Gribaudo. 2006. Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. *Eur. J. Plant Pathol*., 114(4): 397-404.

Grudzinska, M., E. Solarska, A. Czubacka, M. Przybys and A. Fajbus. 2006. Elimination of *Hop latent viroid* from hop plants by cold treatment and meristem tip culture. *Phytopathol. Pol*., 40: 21-30.

Habili, N. 2017. Apscaviroids infecting grapevine. In: *Viroids and satellites* (pp. 251-262). Academic Press.

Hammond, R.W. 2017. Seed, pollen, and insect transmission of viroids. In: *Viroids and satellites* (pp. 521-530). Academic Press.

Hemmati, A.S. and E. Dorani. 2023. Effect of various phenol inhibitors and explant size on meristem culture in the Gavieta variety of Fragaria ananassa. *J. Plant Physiol. Breed*., 13(2): 1-13.

Hu, G.J., Y.F. Dong, Z.P. Zhang X.D. Fan and R.J. Fang. 2021. Elimination of grapevine fleck virus and grapevine rupestris stem pitting-associated virus from *Vitis vinifera* 87-1 by ribavirin combined with thermotherapy. *J. of Integrative Agri.*, 20(9): 2463-2470.

Iandolino, A.B., F. Goes da Silva, H. Lim, H. Choi, L.E. Williams and D.R. Cook 2004. High-quality RNA, cDNA, and derived EST libraries from grapevine (*Vitis vinifera* L.). *Plant Mol. Biol. Rep*., 22(3): 269-278.

Ilyas, S., M. Khalid and S. Naz. 2019. Microshoot tip culture therapy for disease elimination in different varieties of grapes cvs. Princess seedless and Golden Italian Musact in Pakistan. *The J. Anim. Plant. Sci.*, 29(6): 1733-1742.

Jiang, D., S. Peng, Z. Wu, Z. Cheng and S. Li. 2009a. Genetic diversity and phylogenetic analysis of *Australian Grapevine Viroid* (AGVd) isolated from different grapevines in China. *Virus Genes*, 38(1): 178-183.

Jiang, D.M., R. Guo, Z.J. Wu, H.Q. Wang and S.F. Li. 2009b. Molecular characterization of a member of a new species of grapevine viroid. *Archiv. Virol*., 154: 1563-1566.

Karimpour, S., G. Davarynejad, M. Zaki Aghl and M.R. Safarnejad. 2020. Optimization of plant growth regulators for *In vitro* shoot proliferation of apple cv. 'Abbasi' using

response surface method. *J. Plant Physiol. Breed.*, 10(1): 111-125.

Karimpour, S., G. Davarynejad, M. Zakiaghl and M.R. Safarnejad. 2025. *In vitro* thermotherapy and thermo-chemotherapy approaches to eliminate some viruses in *Pyrus communis* L. cv. Natanz. *J. Agri. Sci. Tech.*, 22(6): 1645-1653.

Kim, S.H., M. Zebro, D.C. Jang, J.E. Sim, H.K. Park, Y.K. and S.M. Park 2023. Optimization of plant growth regulators for *In vitro* mass propagation of a disease-free 'Shine Muscat'grapevine cultivar. *Mol. Biol.*, 45(10): 7721-7733.

Krishnamurthy, K.V. and B. Bahadur. 2015. Genetics of flower development. In: *Plant Biol. and Biotech.: Volume I: Plant Diversity, Organization, Function and Improvement* (pp. 385-407). New Delhi: Springer India.

Mahmood, M., S. Naz, A. Gentili, A. Taglienti, A. Ahmad and F. Faggioli. 2022. First survey of twelve grapevine viruses in south east region of Pakistan. *Pak. J. Agri. Sci.*, 59(5): 787.

Maliogka, V.I., F.G. Skiada, E.P. Eleftheriou and N.I. Katis. 2009. Elimination of a new ampelovirus (GLRaV-Pr) and *Grapevine rupestris stem pitting associated virus* (GRSPaV) from two *Vitis vinifera* cultivars combining *In vitro* thermotherapy with shoot tip culture. *Sci. Horti*, 123(2): 280-282.

Markovic, Z., D. Preiner, D. Stupic, Z. Andabaka, S. Simon D. Voncina and F. Engelmann. 2015. Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.). *VITIS-J. Grapevine Res.*, 54: 247-251.

Markovic, Z., P. Chatelet, I. Sylvestre, J.K. Kontic and F. Engelmann. 2013. Cryopreservation of grapevine (*Vitis vinifera* L.) *In vitro* shoot tips. *Cent. Europ. J. Biol.*, 8(10): 993-1000.

Martelli, G. P. 2017. An overview on grapevine viruses, viroids, and the diseases they cause. *Grapevine viruses: Molecular Biology, Diagnostics and Management*, 31-46.

Martin, D., F. Grab, C. Grose, L. Stuart, C. Scofield, A. McLachlan and T. Rutan. 2020. Vintage by vine interactions most strongly influence Pinot noir grape composition in New Zealand: This article is published in cooperation with the XIIIth International Terroir Congress November 17-18 2020, Adelaide, Australia. Guest editors: Cassandra Collins and Roberta De Bei. *Oeno One*: 54(4): 881-902.

Miljanić, V., D. Rusjan, A. Skvarc, P. Chatelet and N. Stajner. 2022. Elimination of eight viruses and two viroids from preclonal candidates of six grapevine varieties (*Vitis vinifera* L.) through *In vivo* thermotherapy and *In vitro* meristem tip micrografting. *Plants*, 11(8): 1064.

Moisseyev, R., V. Kostyukova, A. Pozharskiy, A. Mendybayeva and D. Gritsenko. 2025. First report of *Grapevine Yellow Speckle Viroids* and *Hop Stunt Viroid* in *Vitis vinifera* in Kazakhstan. *Plant Dise.*, PDIS-04.

Morgan, S. W., D.A. Read, J.T. Burger and G. Pietersen. 2023. Diversity of viroids infecting grapevines in the South African *Vitis* germplasm collection. *Virus Genes*, 59(2): 244-253.

Mukherjee, P., N. Husain, S.C. Misra and V.S. Rao. 2010. *In vitro* propagation of a grape rootstock, deGrasset (*Vitis champinii* Planch.): Effects of medium compositions and plant growth regulators. *Sci. Hort.*, 126(1): 13-19.

Nanekar, S., S.J. Patil, S.B. Bhondave, O.L. Mahajan, H.N. Markadand S.K. Holkar. 2025. Diagnosis, diversity, and management of grapevine viruses. In: *Detection, Diagnosis and Management of Air-Borne Diseases in Agricultural Crops* (pp. 209-234).

Perez-Caselles, C., L. Burgos, E. Yelo, L. Faize and N. Alburquerque. 2025. Production of HSVd-and PPV-free apricot cultivars by *In vitro* thermotherapy followed by meristem culture. *Plant Method*, 21(1): 23.

Perrone, I., W. Chitarra, P. Boccacci and G. Gambino. 2017. Grapevine–virus–environment interactions: An intriguing puzzle to solve. *New Phytol.*, 213(3): 983-987.

Rajesh, S., S. Meena, T. Radhamani, P. Sivakumar, S. Shenbagavalli, L. Srimathipriya and J. Rajangam. 2024. *In vitro* culture techniques for disease free propagules production in garlic (*Allium sativum*), a spicy vegetable with therapeutic characteristics. *Appl. Ecol. Environ. Res.*, 22(4): 2941-2957.

Rasool, S. and S. Naz. 2024. Detection and elimination of *grapevine leafroll-associated viruses* (GLRaVs) from *Vitis vinifera* cultivars of Pakistan by meristem tip culture. *Eur. J. Plant Pathol.*, 168(4): 697-707.

Rasool, S., S. Naz and A. Ahmad. 2023. First record of *grapevine yellow speckle viroid-2* infecting grapevines in Pakistan. *J. Plant Pathol.*, 105(3): 1213-1213.

Ren, F., S. Qiao, X. Fan, G. Hu, Z. Zhou and Y. Dong. 2025. Impacts of three virus combinations on grape composition and fruit quality of *Vitis vinifera* Kyoho. *Hort. Plant J.*, 11(1): 446-450.

San Pedro, T., R. Peiro, J. Villanova, A. Olmos and C. Gisbert. 2017. *In vitro* propagation of *Vitis vinifera* L. cv.'Monastrell'. *Elect. J. Biotech.*, 27: 80-83.

Shatnawi, M., G. Anfoka, R. Shibli, M. Al-Mazra'awi, W. Shahrour and A. Arebiat. 2011. Clonal propagation and cryogenic storage of virus-free grapevine (*Vitis vinifera* L.) via meristem culture. *Turk. J. Agri. Fores.*, 35(2): 173-184.

Shoubra, H. 2022. Research article production of virus-free rose plants using meristem-tip culture and *In vitro* thermotherapy. *Pak. J. Biol. Sci.*, 25(2): 160-167.

Skiada, F. G., V.I. Maliogka, N.I. Katis and E.P. Eleftheriou. 2013. Elimination of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) from two *Vitis vinifera* cultivars by *In vitro* chemotherapy. *Eur. J. Plant Pathol.*, 135(2): 407-414.

Tang, W. K., X.P. Lu, W.P. Zhang, Z.C. Tang, L.W. Qu and J.W. Li. 2024. HSVd elimination from tomato using cryotherapy combined with exogenous HSVd-targeting ds-sRNA Application. *Sci. Hort.*, 337: 113463.

Wang, H. 2025. Medical Benefits and Polymer Applications of Grapes. *Polymers*, 17(6): 750.

Wang, Y. and Y. Jiao. 2018. Axillary meristem initiation—a way to branch out. *Curr. Opin. Plant Biol.*, 41: 61-66.

Wondimu, T., T. Feyissa and G. Bedadav. 2012. Meristem culture of selected sweet potato (*Ipomoea batatas* L. Lam.) cultivars to produce virus-free planting material. *J. Hort. Sci. Biotech.*, 87(3): 255-260.

Zhang, Z., S. Peng, D. Jiang, S. Pan, H. Wang and S. Li. 2012. Development of a polyprobe for the simultaneous detection of four grapevine viroids in grapevine plants. *Eur. J. Plant Pathol.*, 132(1): 9-16.