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Clonal propagation and cryogenic storage of virus-free grapevine (*Vitis vinifera* L.) via meristem culture

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Abstract: A protocol for production of virus-free *Vitis vinifera* using meristem culture was developed. Meristems (0.1-0.2 mm) of *V. vinifera* infected with grapevine fanleaf virus and grape leafroll associated viruses were excised from 1-year-old growing vines. Shoot tips were cultured on half-strength Murashige and Skoog (MS) medium, supplemented with 0.02 mg L^{-1} of benzylaminopurine (BAP) and 0.01 mg L^{-1} of naphthalene acetic acid (NAA). BAP and kinetin resulted in differences in the number of new shoots per explant, shoot height, and number of new leaves per explant. BAP at 0.8 mg L^{-1} gave the highest in vitro multiplication rate, with 5.25 shoots per explant, whereas elongation was greatest in the presence of 0.2 mg L^{-1} of kinetin. Root initiation was tested on an MS medium supplemented with 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg L^{-1} of IBA (indole-3-butyric-acid), IAA (indole-3-acetic-acid), or NAA. Maximum root number was achieved using 0.6 mg L^{-1} of IBA. A survival rate of 95% was achieved when rooted explants were acclimatized ex vitro in a mixture of 1 soil: 1 perlite: 1 peat. Acclimatized plants grew in the greenhouse and were maintained as virus-free plants. Visual inspections as well as results of RT-PCR, using virus-specific oligonucleotide primers, showed that plants developed in vitro were free from grapevine fanleaf virus, grape leafroll associated virus-1, and grape leafroll associated virus-3 infections. Shoot tips from plantlets grown in vitro were cryopreserved by vitrification. Shoot tips were treated in a 2 mL cryotube filled with a solution of 5% (w/v) DMSO, 5% (w/v) glycerol, and 5% (w/v) sucrose at 25 °C for 20 min, then dehydrated with 1 mL of modified vitrification solution 2 (MPVS2) at 0 °C for 40 min. Maximum regrowth (55%) was obtained after cryogenic storage with cultures exposed to MPVS2 for 40 min at 0 °C. Cryopreserved shoot tips, after being warmed, resumed growth within 7 days and developed shoots directly without intermediate callus formation.

Key words: Clonal propagation, cryogenic storage, grapevine, in vitro root formation, meristem cultures, virus-free grapevine, *Vitis vinifera*

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Introduction

Grapevine (*Vitis vinifera* L.) is a widely distributed plant worldwide, and the demand for grape products has increased dramatically. Viral diseases constitute a major hindrance to the development and profitable production of viticulture (Al-Tamimi 1998; Wang et al. 2002). Grapevines diseases are caused by intracellular pathogens of different natures, among which plant viruses are the most important (Martelli 1989; Shahrour 2004). However, not all viruses cause severe damaged, as they represent occasional contaminations of viruses in specific environments (Martelli 2003). Plants infected with grape viral diseases show less vigorous growth and delayed bud opening, and may decline and die within a few years. On the other hand, viruses that cause negative effects on quality and quantity of the yield are grapevine fanleaf virus (GFLV), rugose wood virus, grape leafroll associated viruses (GLRaVs), and grape fleck virus (GFKV) (Hadidi et al. 1998; Martelli 2003; Shahrour 2004).

GFLV is one the most serious and devastating grapevine virus diseases. Sensitive cultivars show rapid decline and low quality of fruit and yield. The most damaging consequence of GFLV is a severe reduction in fruit setup that can greatly reduce the yield (Shahrour 2004). Infected vines show misshapen leaves with the appearance of an open fan, thus the name "fanleaf." Other visible symptoms include yellowing of the leaves (mosaic), bright yellow bands near veins (vein banding), abnormal branching, and short internodes. Vines affected by GFLV have poor fruit set, and the fruit ripens irregularly. A total of 9 viruses were reported to be associated with grapevine leafroll disease. Seven of these viruses (GLRaV-1, -3, -4, -5, -6, -8, and -9) belong to the genus *Ampelovirus*, while GLRaV-2 belongs to the genus *Closterovirus* of family *Closteroviridae*. Disease symptoms caused by GLRaV-3 appear in *Vitis vinifera*, but they are latent in almost all American *Vitis* plants. Diseased leaves are thicker than normal and brittle, with margins rolled downwards and discolored (Martelli 1993). These symptoms are usually associated with yield losses of 20%-40% (Woodrum et al. 1984). Numerous studies have demonstrated transmission of GLRaV-3 in vineyards by several species of mealybugs. GLRaV-1 can be transmitted by the soft scale *Parthenolecanium corni* and the mealybugs *Heliococcus bohemicus* and *Phenacoccus aceris* (Sforza and Greif 2000; Sforza et al. 2003). In Jordan, although grapevines are widely grown, information on the distribution of grapevine viruses is limited. Recently, GFLV, GLRaV-3, and grapevine virus A were detected and characterized at the molecular level (Anfoka et al. 2004a; Anfoka et al. 2004b; Shahrour 2004).

Conservation of virus-free plants is time consuming, expensive, and implies the risk of lost valuable material. To reduce this adverse effect, cryogenic storage is an ideal tool for long-term conservation of virus-free plants, since it would probably provide a reproducible source of in vitro culture. In vitro plants have been employed for cryopreservation, including calluses, shoot tips, protoplasts, and cell suspension cultures, as they contain high amounts of cellular water and are thus extremely sensitive to freezing injury. Cryogenic storage of shoot tips in liquid nitrogen has therefore been recognized for the long-term storage of living plant material (Bekheet et al. 2007; Shatnawi et al. 2007), in which cell division and metabolic processes cease (Sakai et al. 2000). One of the techniques used to achieve dehydration is the vitrification method. The key to successful vitrification is the acquisition of osmotolerance and the mitigation of any injurious effects during the dehydration process (Sakai et al. 2000). Successful vitrification requires application of a highly concentrated solution to prevent any injury associated with either chemical toxicity or excess osmotic stress during dehydration.

The control of grapevine viruses is dependent upon the effectiveness of clean stock programs, where virus-free propagation material is used in nurseries and vineyards. According to Wang et al. (2002), meristem culture and thermotherapy are the most often employed methods for obtaining virus-free grapevine plants. Therefore, attempts have been made in this study to develop efficient protocols for the clonal propagation and cryogenic storage of a virus-free local Jordanian cultivar (Salty Kodari) of *V. vinifera* from a virus-infected mother stock.

Materials and methods

Establishment of in vitro culture

Shoot tips (2-5 mm) of grapevine (*V. vinifera* cv. 'Salty Kodari') were collected from GFLV-, GLRaV-1-, and GLRaV-3-infected plants that were grown in the field station of the Agriculture Research Center of the Jordan University of Science and Technology (JUST), Irbid, Jordan, as described by Wang et al. (2002). Shoot tips were then immersed in tap water and kept at a temperature of 24 ± 2 °C by day and 18 ± 2 °C by night for 1 day. Shoot tips were sterilized in 5% Clorox (5.25 sodium hypochlorite) plus 0.1% Tween-20 (surfactant) for 10 min, and then rinsed 3 times with sterile distilled water (for 5 min each time) in a laminar airflow cabinet. Meristems (0.1-0.2 mm) were then isolated under a binocular microscope (Shatnawi et al. 1999) and inoculated on half-strength MS medium (Murashige and Skoog 1962). The medium was supplemented with 0.02 mg L^{-1} of BAP, 0.01 mg L^{-1} of NAA, 30 g L^{-1} of sucrose, and 8.0 g L^{-1} of agar agar. A medium pH was adjusted to 5.8-5.9 prior to sterilization. Shoot tips were kept in the dark for at least 2 days, then moved to the growth chamber (24 ± 2 °C) under a 16:8 h photoperiod (photosynthetic flux, 40-50 µmol $m^{-2}s^{-1}$). After that, shoot tips were transferred to a new MS medium, supplemented with the same hormones, and left to grow in the culture. Four weeks later, microshoots were transferred to fresh media before in vitro multiplication. Subculturing was performed every 4 weeks to establish a massive mother stock culture before initiating the experiments. Figure 1 outlines the general experimental approach for this study.

Effect of cytokinin on shoot proliferation

Microshoots were subcultured on hormone-free MS medium for 2 weeks to eliminate any carry-over effects of the basic cytokinin (Shibli et al. 1997). Microshoots (10 mm in length) were then subcultured on fresh MS medium. For shoot proliferation, microshoots were subcultured on MS medium supplemented with either BAP or kinetin at 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg L^{-1} with the addition of 0.01 mg L^{-1} of IBA. For each replicate, 50 mL of the medium was dispensed into 250 mL culture vessels. Each treatment consisted of 15 replicates, each with 3 microshoots, and each experiment was repeated twice. Culture conditions were maintained as described above.

Effects of auxins on root formation

Microshoots were subcultured on hormone-free MS medium for 2 weeks to eliminate any carry-over effects of the cytokinin. Microshoots (10 mm in length) were then subcultured on MS medium supplemented with 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg L^{-1} of IBA, IAA, or NAA. For each replicate, 50 mL of the medium was dispensed into 250 mL culture vessels. Each treatment consisted of 15 replicates, each with 3 microshoots, and each experiment was repeated twice. Culture conditions were identical to those described previously. Four weeks later, data were collected on shoot lengths, number of proliferated shoots, number of roots/explants, root lengths, and the percentage of shoots developing roots.

Ex vitro acclimatization

Flasks were opened for 2-3 days to reduce the humidity and to let the plantlets develop a surface waxy layer to prevent water loss (Shibli et al. 1997). Plantlets were gently removed from the culture flask and rinsed in water to ensure that the agar was removed. Acclimatized plantlets were transferred to pots containing a mixture of 1 soil: 1 perlite: 1 peat and grown under greenhouse conditions, at 24 ± 2 °C by day and 18 ± 2 °C by night. Immediately after potting, plantlets were misted with water and the humidity was maintained close to 100%. Plants were covered with a plastic cover for 2 weeks, and then the humidity was progressively decreased by manually opening the plastic. This allowed a gradual acclimatization to occur over a periods of 4 weeks. After 6 weeks, the surviving plants were counted. Data were collected for the percentage of survival of plants that were acclimatized ex vitro.

RNA extraction

Total RNA was extracted from leaves and petioles of 10 grapevine seedlings using the Total RNA Isolation System Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. To prevent contamination, all materials, water, and solutions were treated with 0.2% diethylpyrocarbonate (DEPC). In summary, 50 mg of grapevine tissues were ground by mortar and pestle after being frozen in liquid nitrogen (LN). Ground tissues were transferred to 600 µL of denaturing solution, and 60 µL of 2.0 M sodium acetate (pH 4.0)

was added. After that, 600 µL of phenol, chloroform, and isoamyl alcohol were added in a ratio of 25:24:1, and the solution was mixed by inverting it 3-5 times and shaking vigorously for 10 s. The mixture was then chilled on ice for 15 min and centrifuged at 14,000 rpm for 20 min. The supernatant was removed to a new tube and the total RNA was precipitated by isopropanol. The RNA pellet was dissolved in TE buffer and kept at -80 °C for further use.

Detection of GLRaV-3, GLRaV-1, and GFLV by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extracted from grapevine tissues developed in vitro was used to detect GLRaV-3, GLRaV-1, and GFLV infection using specific primer pairs (Table 1). One-step RT-PCR was performed in a final volume of 50 µL of the following PCR mixture: 1× M-MLV buffer, 1× Taq polymerase buffer, 0.2 mM dNTPs mixture, 1 mM $MgSO_4$, and 1 µM of each primer to each virus's gene, separately (Table 1). Then 0.1 U μL^{-1} of Taq DNA polymerase and 0.1 U μL^{-1} of M-MLV reverse transcriptase were added to 5.0 µL of RNA. To allow cDNA production, tubes were incubated at 37 °C for 1 h and then PCR amplification was performed in a programmable thermal controller (model PTC-200, MJ Research Inc., Watertown, MA, USA), as described in Table 1. Aliquots (10 µL each) of PCR products were resolved electrophoretically on a 1.5% agarose gel using 0.5× TBE buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 µg mL^{-1}) (Promega). DNA fragments were visualized with a UV transilluminator and photographed with the gel documentation system (Gel Doc 2000, BIO-RAD, USA). A DNA molecular weight marker, a 100 bp DNA ladder (Fermentas, USA), was used to determine the size of the amplified fragments.

Cryogenic storage

Shoot tips free of virus were excised from in vitro plants. Excised shoot tips (2-3 mm) were pretreated on a solid MS medium containing 0.3 M sucrose for 1 day in the dark. Shoot tips were then pretreated with a loading solution of 5% (w/v) DMSO, 5% (w/v) glycerol, and 5% (w/v) sucrose, the composition of which was hormone-free MS, for 20 min at 0 °C. The loading solution was then removed and replaced with one of the following vitrification solutions:

1. PVS2: 30% (w/v) glycerol, 15% (w/v) DMSO, 15% ethylene glycol + 14% (w/v) sucrose in full-strength MS medium (Sakai et al. 1990).

2. MPVS2: 15% (w/v) glycerol, 20% (w/v) DMSO, 15% ethylene glycol + 14% (w/v) sucrose in full-strength MS medium.

Table 1. Primer pairs used to detect grapevine viruses by RT-PCR.

Primer	References	Sequence (5′ to 3′)	Virus	Target gene	Size (bp)	RT-PCR cycling conditions
C547 **H229**	Minafra and Hadidi 1994	TTAACTTGACGGATGGCACGC ATAAGCATTCGGGATGGACC	GLRaV-3	*RdRp*	340	94 °C/5 min, 15X (94 °C/50 s, 54 °C/1 min, 72 °C/1 min), 20X (94 °C/50 s, 46 °C/70 s, 72 °C/1 min), 72 °C/10 min
CPv **CPc**	Fazeli and Rezaian 2000	TTGGATCCGCTAGCGTTATAT CTCAAAATGATAATG AGTAAGCTTTTATTACACCTT AAGCTCGCTAGTATTC	GLRaV-1	*CP*	966	95 °C/4 min, 35X (94 °C/1 min, 56 °C/2 min, 72 °C/2 min), 72 °C/7 min
C2647 **H2042**	Fattouch et al. 2001	GTGAGAGGATTAGCTGGT AGCACTCCTAAGGGCCGT	GFLV	*CP*	606	92 °C/5 min, 35X (92 °C/30 s, 48 °C/45 s, 72 °C/1 min), 72 °C/10 min

Shoot tips were exposed to the vitrification solutions for varying durations, 0, 20, 40, 80, 120, 160, or 200 min, at 0 °C. The vitrification solution was replaced once at the end of the exposure time and then the tips were suspended in 0.5 mL of fresh vitrification solution in the cryotube immediately before immersion in LN for a period of 1 h to 1 day. After storage in LN, cryotubes were thawed rapidly in a water bath at 45 °C for 2 min. After thawing, the vitrification solution was drained from the cryotubes and replaced with MS liquid medium supplemented with 14% (w/v) sucrose, in which the tips were washed for 15-20 min (Niino et al. 1992). Frozen and unfrozen shoot tips were then transferred to a solid hormone-free MS medium supplemented with 3% (w/v) sucrose. Cultures were incubated in the dark for 1 week and then transferred to standard culture conditions at 24 ± 2 °C. For regrowth assessments, regrowth was defined as the percentage of shoot tips resuming growth (becoming green) 8 weeks after plating out. Recovery of shoot tips was observed at weekly intervals. Surviving plantlets were then subcultured into a hormone-free MS medium every 3 weeks.

Statistical analysis

Data were subjected to ANOVA. Differences between individual means were determined by the least significant differences (LSD) test at the 0.05 level of probability. Data were analyzed using STATISTICA (Statsoft, Inc. 1995).

Results

A key aim of this study was to develop virus-free grapevine plants using the meristem culture technique. Therefore, meristems were obtained from grapevine plants infected with 3 grapevine viruses (GLRaV-3, GLRaV-1, and GFLV) and used as starting materials. The optimization process, incorporating the testing of a series of plant growth hormones to identify the ideal combination for shoot growth, is demonstrated in Figure 1. A protocol has been

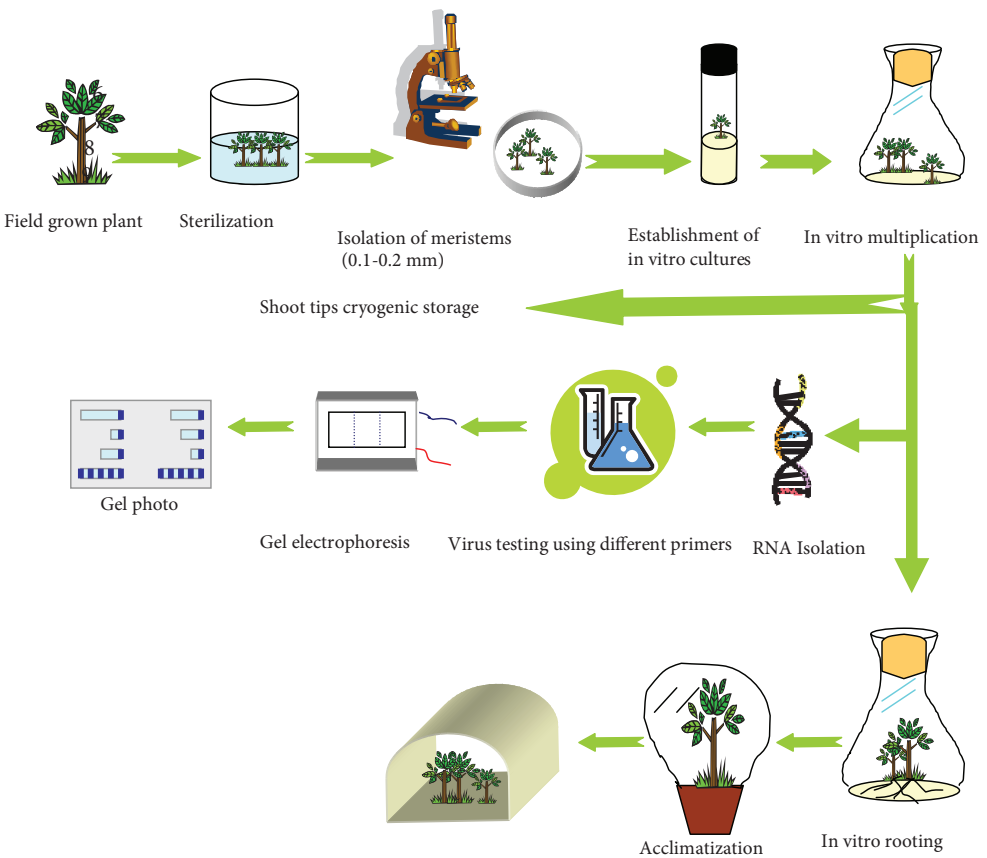

Figure 1. Outline of the research project of this study.

established for inducing a high frequency of plant propagation from single explants of *V. vinifera* using meristem culture.

In vitro micropropagation

Table 2 shows the effect of BAP and kinetin on the in vitro growth of *V. vinifera*. Cytokinin stimulated cell division and released lateral bud dormancy. Shoot multiplication had elongation on media containing BAP (Figure 2a). No statistical significant differences were detected in the number of new leaves with the supplementation of kinetin. The supplementation of BAP had notable effects on the number of leaves, where the maximum number of new leaves obtained with a supplement of 0.8 mg L^{-1} of BAP was not significant from 1.0 mg L^{-1} of BAP (Table 2). Maximum shoot length (28.5 mm) was obtained with the supplementation of 0.2 mg L^{-1} of kinetin. No significant differences occurred in the number of new shoots per explant in response to different kinetin concentrations. However, there was a statistically significant difference in the number of new shoots in response to different BAP concentrations (Table 2). The highest number of new shoots (5.25) was obtained when the media were supplemented with 0.8 mg L^{-1} of BAP.

In vitro root formation

V. vinifera responded differently to media supplemented with IBA, IAA, and NAA (Table 3). There was no significant difference in the number of new shoots produced per explant. On the other hand, there was a significant difference in the number of roots per explant, as well as the root length. Additionally, auxins did not increase the formation of new shoots significantly. After 4 weeks of the growth period, the percentage of root formation was up to 80% in the medium supplemented with IBA at 0.2-0.4 mg L^{-1}. Shoot length reached its maximum with 0.6 mg L^{-1} of IBA, which was not significant when compared with a further increase in IBA (0.8-1.0 mg L^{-1}). However, the number of roots per explant increased significantly with an increasing IBA concentration, to 0.6 mg L^{-1}; this was not significantly different when compared with higher IBA concentrations (0.8 or 1.0 mg L^{-1}). An increased IAA concentration increased root number and root length. Maximum root length was obtained with 0.6-0.8 mg L^{-1} of NAA.

Table 2. Influence of BAP or kinetin on number of new leaf, shoot length, number of new shoots per explant and callus formation of *in vitro* grown *V. vinifera*.

Growth regulator (mg L^{-1})	Number of new leaves per explant	Shoot length (mm)	Number of new shoots per explant	Callusing
		BA		
0.0	4.25 a	20.75 c	1.00 a	-
0.2	4.50 a	18.25 bc	2.30 b	+
0.4	6.25 b	16.25 ab	2.40 b	+++
0.6	7.50 c	16.00 a	2.40 b	+++
0.8	9.75 d	14.00 a	5.25 d	++++
1.0	9.25 d	14.25 a	4.00 c	++++
		Kinetin		
0.0	4.25 a	20.75 c	1.00 a	-
0.2	4.50 a	28.50 d	1.20 a	+
0.4	4.50 a	26.75 d	1.30 a	+
0.6	4.30 a	18.25 bc	1.30 a	+
0.8	5.25 a	19.25 c	1.35 a	+
1.0	4.25 a	17.25 b	1.30 a	++

Values represented means; each treatment consisted of 15 replicates, with 3 microshoots each treatment, each experiment repeated twice. Means within a column with the same letter are not significantly different based on least significant difference (LSD) at 0.05 level of probability. (-) = no callus. (+, ++, +++, ++++) = (callus with 2-4, 4-6, 6-8, 8-10, mm) diameter, respectively.

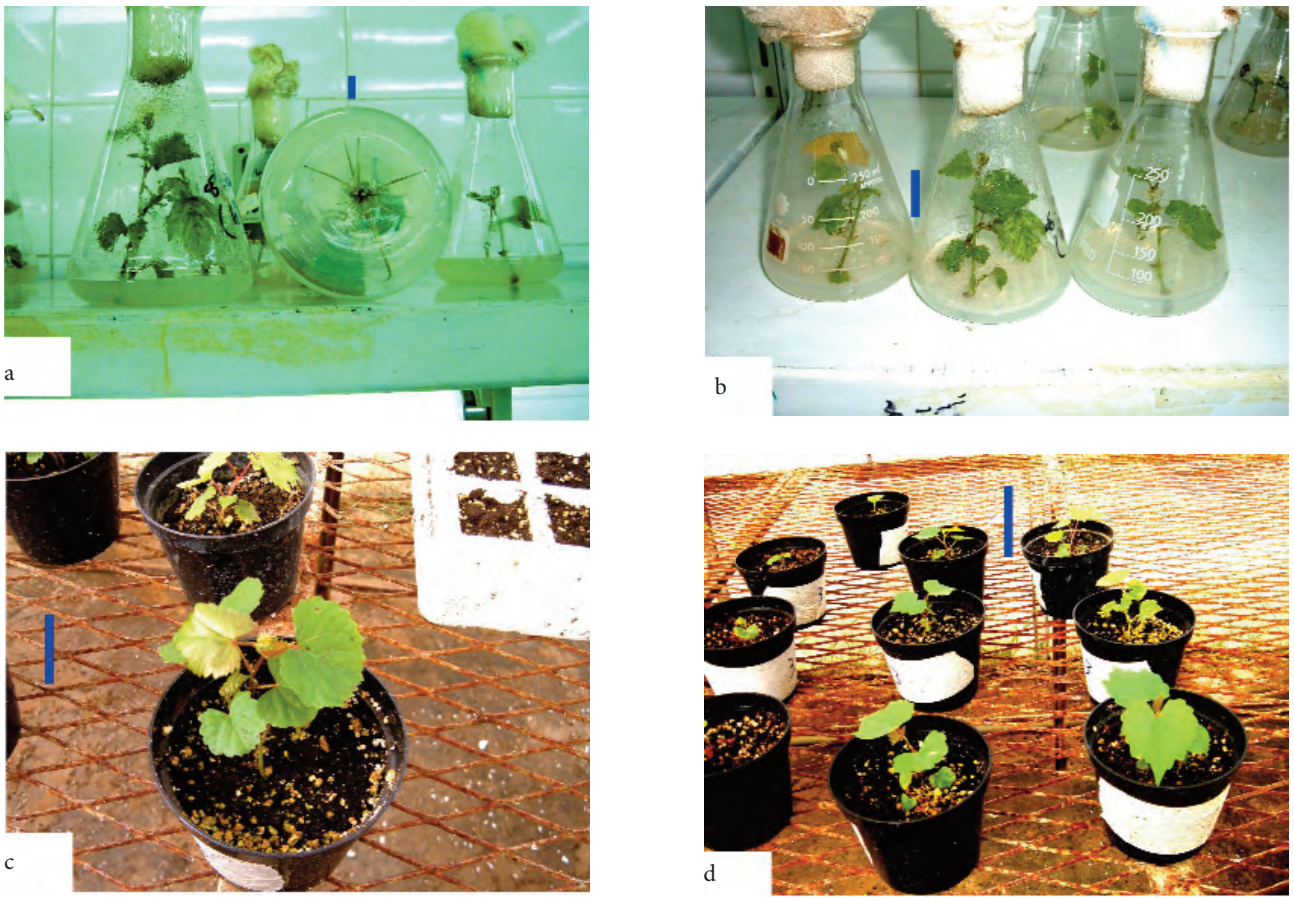

Figure 2. a) Multiple shoot formation of *V. vinifera* on MS medium + 0.6 mg L^{-1} of BAP after a 4-week growth period. b) In vitro root formation of *V. vinifera* on MS medium + 0.4 mg L^{-1} of NAA after a 4-week growth period. c) *V. vinifera* transplanted plantlets growing in the greenhouse after a 6-week acclimatization period. d) *V. vinifera* transplanted plantlets growing in the greenhouse after a 10-week acclimatization period. Bar represents 5 cm.

In vivo acclimatization

Transfer of in vitro rooted plantlets to greenhouse conditions is a critical operation. Rooted plantlets, when moved to the acclimatization conditions, showed 95% survival, and the plants were healthy, demonstrating a good green color and rapid growth.

Detection of grapevine viruses by RT-PCR

The expected sizes (340, 966, and 606 bp) of GLRaV-3, GLRaV-1, and GFLV, respectively, could only be detected when RNA was extracted from infected grapevine tissues. No bands specific to GLRaV-3, GFLV, or GLRaV-1 were detected from the tissues obtained from plants developed in in vitro culture (Figure 3).

Cryogenic storage of shoot tips

It was found that exposure to the vitrification solutions decreased the regrowth of unfrozen tips (Table 4), and regrowth decreased significantly with increased exposure time. However, the percentage of shoot formation of unfrozen shoot tips was generally higher than those of cryopreserved tips. The percentage of shoot formation after storage in liquid nitrogen ranged from 1.7%-55.3%, whatever the components of the freezing solution were. Statistical analysis also indicated that, for each vitrification solution, there was a significant interaction between shoot formation (in frozen and unfrozen treatments) and time of exposure to the vitrification solution.

Table 3. Influence of IBA, IAA and NAA on number of shoots, shoot length, number of root and root length of *in vitro* grown *V. vinifera*.

Growth regulator (mg L^{-1})	Number of shoots per explant	Shoot length (mm)	Number of roots per explant	Root length (mm)	Root formation (%)
			IBA		
0.0	1.00	21.35 a	0.00 a	0.00 a	**0.0**
0.2	1.20	46.75 c	3.24 b	34.5 b	**80**
0.4	1.20	47.76 c	4.56 c	33.4 b	**80**
0.6	1.20	57.80 d	6.78 d	35.8 b	**95**
0.8	1.30	45.80 c	5.84 d	29.3 b	**100**
1.0	1.30	55.40 d	5.80 d	28.9 b	**100**
			IAA		
0.0	1.00	21.35 a	0.00 a	0.00 a	**0.0**
0.2	1.20	38.35 b	2.34 b	44.5 b	**90**
0.4	1.20	46.45 c	2.45 b	45.9 b	**90**
0.6	1.20	45.76 c	2.96 b	47.7 b	**100**
0.8	1.25	39.45 b	3.20 b	49.7 b	**97**
1.0	1.20	44.56 c	3.25 b	47.8 b	**100**
			NAA		
0.0	1.00	21.35 a	0.00 a	0.00 a	**0.0**
0.2	1.20	36.56 b	3.24 b	33.5 b	**90**
0.4	1.25	48.67 c	3.30 b	45.8 b	**95**
0.6	1.25	58.89 d	3.27 b	55.8 c	**100**
0.8	1.25	45.76 c	3.30 b	56.9 c	**100**
1.0	1.30	49.34 c	3.35 b	45.8 b	**100**

Values represented means; each treatment consisted of 15 replicates, with 3 microshoots each treatment, each experiment repeated twice. Means within a column of the three hormones tested, with the same letter are not significantly different based on least significant difference (LSD) at 0.05 level of probability.

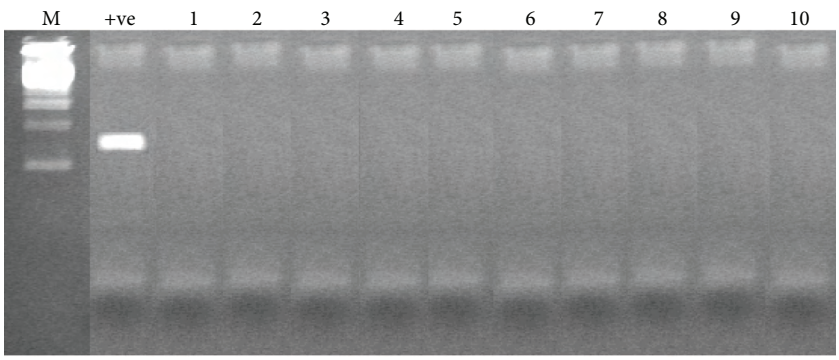

Figure 3. Agarose gel (1.5%) electrophoresis of the coat protein gene of grapevine virus GLRaV-3 amplified by RT-PCR using the primer C2647; H2042. Total RNA extracted from *V. vinifera* cv. 'Salty Kodari' grapevine grown in vitro. Lines: 1-10, *V. vinifera* grown in vitro; M, Molecular weight 1 Kb marker; +ve, positive control of GLRaV-3.

Table 4. Effect of exposure time with PVS2 and MPVS2 at 0 °C, on regrowth percentage of non-cryopreserved and cryopreserved shoot tips of *V. vinfera*. Shoot tips were loaded with a mixture of (5% (w/v) DMSO, 5% (w/v) glycerol and 5% (w/v) sucrose) for 20 min at 0 °C.

Exposure duration (min)	Vitrification solution	
	Regrowth % of unfrozen shoot tips	
	PVS2	***MPVS2***
0	100 ± 0.0 e	100 ± 0.0 i
20	100 ± 0.0 e	94.0 ± 2.5 i
40	90.6 ± 5.2 e	94.0 ± 3.7 i
80	77.2 ± 5.2 d	67.0 ± 4.3 h
120	44.3 ± 4.9 c	36.5 ± 2.5 e
160	19.5 ± 3.1 b	24.0 ± 3.2 c
200	8.3 ± 3.3 a	6.50 ± 3.1 ab
	Regrowth % of frozen shoot tips	
0	0.0 ± 0.0 a	0.0 ± 0.0 a
20	20.8 ± 3.7 b	26.3 ± 2.3 d
40	47.3 ± 7.2 c	55.3 ± 3.3 g
80	24.0 ± 2.2 b	44.1 ± 4.2 f
120	19.3 ± 0.4 b	12.0 ± 3.6 b
160	9.5 ± 4.2 a	13.3 ± 2.1 b
200	0.0 ± 0.0 a	2.7 ± 1.7 a

Means within a column with the same letter are not significantly different based on LSD test at $P \leq 0.05$. Means ± standard error, n = 3. Ten shoot tips were tested for each replicate. Regrowth was defined as the percentage of cryopreserved shoot tips resuming normal growth 8 weeks after plating out.

Regrowth of noncryopreserved tips remained at 100% with an exposure time of 20 min, but was significantly reduced after 80 min. A regrowth rate of unfrozen shoot tips of 77% was obtained following 80 min of exposure to PVS2 solution, while a regrowth rate of 67% was obtained after exposure for 60-90 min to MPVS2. Frozen tips treated with MPVS2 for 40 min showed a regrowth rate of 55%, but longer exposures to MPVS2 caused a significant reduction in regrowth (Table 4). At the 2 longest exposures, higher survival rates for frozen tips occurred in treatments with MPVS2. The maximum regrowth rate using PVS2 was 47%, obtained following 40 min of exposure to PVS2 solution. Surviving vitrified shoot tips remained green after thawing and plating out on hormone-free MS, and they started to grow after 3-4 weeks. Recovery of shoot tips occurred directly, without callus formation.

Discussion

Micropropagation of *V. vinifera* by in vitro culturing has the potential for producing large numbers of plants within a relatively short period of time. The number of new shoots was affected by the concentration of BAP and kinetin over a 4-week growth period (Table 2). There was slight improvement in shoot proliferation when 0.2 mg L^{-1} was used; this is similar to a previous finding by Couselo et al. (2006).

Callusing was present at the microshoot base with supplementation of BAP or kinetin. The biggest calluses (8-10 mm) occurred with 0.8 or 1.0 mg L^{-1} of BAP (Table 2). Similar results have been reported by Pathirana and McKenzie (2005) and Mezzetti et al. (2002). No root formation was recorded in any medium supplemented with cytokinins. Root

formation was diminished or inhibited with the presences of cytokinins. This agrees with previous findings obtained on grapes (Mezzetti et al. 2002; Salunkhe et al. 2004; Pathirana and McKenzie 2005; Couselo et al. 2006). The addition of cytokinin to the medium led to increased leaf numbers, number of new shoots, and shoot length in grapes (Pathirana and McKenzie 2005; Couselo et al. 2006).

Micropropagation protocols must produce plantlets with quality shoots and roots, suitable for transfer to soil conditions (Pierik 1987). However, auxins enhanced the root formation as compared with the control (hormone-free medium), which showed no root formation (Table 3). In this study, plantlets with roots around 10 mm in length were found to be convenient for handling during transplanting (acclimatization) (Figure 2b), because roots longer than 10 mm have a higher probability of accidental damage during handling, which has a substantial adverse effect on the survival of transplanted plants, as described by a previous report (Razdan 1992). The results of this study were very similar to those by Pathirana and McKenzie (2005) on *V. vinifera*. However, variability within the species' response to in vitro rooting growth using different regulators and concentrations has been reported in *Prunus amygdalus* (Shatnawi 2006) and *Pyrus syriaca* (Shibli et al. 1997). Therefore, each species requires trials to optimize a root induction for in vitro propagation. Similar trends have been observed in grapevine (Pathirana and McKenzie 2005; Couselo et al. 2006). Plantlets resumed normal growth in the greenhouse, developing new leaves within 20 days. All surviving plants showed uniform growth, had normal leaf development, and lacked any detectable morphological variation. In the present study, seedlings of Salty Kodari grapevines free from GLRaV-3, GLRaV-1, and GFLV were developed by in vitro culturing. This is in accordance with results of previous studies that showed the use of in vitro culture to produce virus-free grapevine plants (Gribaudo et al. 1997; Goussard et al. 1999).

For cryopreservation, PVS2 and MPS2 are essential in achieving a high regrowth rate for cryostorage of *V. vinifera*. However, we observed that MPVS2 produced a higher regrowth rate as compared with PVS2. This is probably due to solute accumulation, reduction of isotonic water content, reduction in vacuolar volumes, and changes in membrane structure (Bekheet et al. 2007). However, an increased DMSO concentration in MPVS2 may cause an increase in the regrowth rate. The beneficial effect of DMSO might be related to some sort of favorable osmotic adjustment that allows cryopreserved shoot tips to recover from the trauma of cryoshock. Increasing the time of exposure to vitrification solutions leads to the increase of solute concentrations inside the vitrification of the cryopreserved plant tissues, eliminating the potential damaging effects of intra- and extracellular ice crystallization.

In this study, shoot tips conserved in LN resumed growth after thawing at levels similar to those cryopreserved for 1 h to 1 day. This result suggests that long-term storage of shoot tips is possible for *V. vinifera* using the methods developed. Hormone-free MS medium was used in this research, in a successful effort to avoid any callus formation and to preserve true-to-type regrowth. Cryopreserved shoot tips generally regenerated into normal plantlets after thawing, and continuous subculture of surviving shoot tips was employed to obtain healthy plantlets. Shoot tips that lost their color completely after exposure to LN were found, in fact, to be dead. Complete color loss after cryopreservation has been reported in previous research as an indication for shoot tip death (Towill 1990; Niino et al. 1992). Color changes of shoot tips may be due to the osmotic stress caused by vitrification solutions (Sakai et al. 1991) or to the synthesis of toxic substances during the combination of stresses that plant tissues face during cryopreservation (Engelmann 1997).

In this study, cryopreserved shoot tips produced phenotypic plantlets identical to those of the controls, and plants regenerated from vitrified shoot tips were morphologically uniform. The methods developed, therefore, appear to be suitable for cryopreservation of shoot tips from stocks of *V. vinifera* grown in vitro. Additional studies need to be undertaken on this species in order to increase recovery of tips after cryopreservation and to standardize protocols. The data of this study showed production of virus-free grapevine (*V. vinifera*) using tissue culture methods. Similar to the results of our study, Abu Shirbi (2001)

demonstrated the value of meristem culture coupled with thermotherapy to get virus-free grapevine from GFLV-infected grapevine. Thus, the possibility of obtaining virus-free plants from plants grown in vitro appears to be promising for the production of virus-free plants, opening the possibility of preserving clean plant material in vitro.

References

Abu Shirbi A (2001) Effect of thermal therapy treatments and meristem tip culture on freeing grapevine infected with grapevine fanleaf virus. Ph.D. Dissertation. University of Jordan, Amman, Jordan.

Al-Tamimi N (1998) Evaluation of the sanitary status of grapevine in Jordan and biological, serological and physiochemical comparison of some GFLV isolates. Master Thesis. Mediterranean Fruit Crop, IAM-Bari, Italy.

Anfoka GH, Shahrour W, Nakhla MK (2004a) Detection and molecular characterization of Grapevine virus A (GVA) in Jordan. Phytopath Med 43: 387-394.

Anfoka GH, Shahrour WE, Nakhla MK (2004b) Detection and molecular characterization of grapevine fanleaf virus and grapevine leafroll-associated virus 3 in Jordan. J Pla Path 86: 203-207.

Bekheet SA, Taha, HS, Saker MM, Solliman ME (2007) Application of cryopreservation technique for in vitro grown date palm (*Phoenix dactylifera* L.) culture. J App Sci Res 3: 859-866.

Couselo JL, Varela P, Rey M (2006) Effect of benzyladenine concentration and double-phase culture system on in vitro multiplication of adult Albarino plants. Am J Ento Vitic 57: 109-112.

Engelmann F (1997) In vitro conservation methods. In: Biotechnology and Plant Genetic Resources (Eds. JA Callow, BV Ford-Lloyd, HJ Newbury). CAB International, Berlin, pp. 119-161.

Fattouch S, M'hirsi S, Acheche H, Marrakchi M, Marzouki N (2001) RNA oligoprobe RT-PCR, a sensitive method for the detection of grapevine fanleaf virus in Tunisia grapevines. Pla Mol Bio Rep 19: 235-244.

Goussard PG, Wiid J, Kasdorf GG (1999) The effectiveness of in vitro somatic embryogenesis in eliminating fanleaf virus and leafroll-associated viruses from grapevines. Afr J Enol Vitic 12: 77-81.

Gribaudo I, Mannini F, Lenzi R (1997) Virus elimination in grapevine cultivars of north-western Italy through meristem culture and *in vitro* thermotherapy. Extended Abstracts, 12th Meeting of ICVG, Lisbon: 165-166.

Fazeli F, Rezaian M (2000) Nucleotide sequence and organization of ten open reading frames in the genome of grapevine leafroll-associated virus and identification of three subgenomic RNAs. J Gen Viro 81: 605-615.

Hadidi A, Khetarpal R, Koganezawa H (1998) Plant virus disease control. The American Phytopathological Society Press, Minnesota, USA.

Martelli G (1989) Infectious diseases of grapevines: nature, detection, sanitation and situation in the Arab countries. Arab J of Pla Prot 7: 219-210.

Martelli G (1993) Rugose of grapevine. In: Graft-transmissible Diseases of Grapevines. Handbook for Detection and Diagnosis (Ed. G Martelli). FAO Publication, Division, Rome, pp. 47-48.

Martelli G (2003) Grapevine virology highlights 2000-2003. 14th ICVG Conference. Bari, Italy.

Mezzetti B, Pandolfini T, Navacchi O, Landi L (2002) Genetic transformation of *Vitis vinifera* via organogenesis. BMC Biotechnology 2: 18.

Minafra A, Hadidi A (1994) Sensitive detection of grapevine virus, A, B or leafroll-associated III from viruliferous mealybugs and infected tissue by cDNA amplification. J Viro Meth 47: 175-188.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Niino T, Sakai A, Enomoto S, Magosi J, Kato S (1992) Cryopreservation of *in vitro*-grown shoot tips of mulberry by vitrification. CryoLetters 13: 303-312.

Pathirana R, McKenzie M (2005) Early detection of grapevine leafroll virus in *Vitis vinifera* using in vitro micrografting. Pla Cell Tiss Org Cul 81: 11-18.

Pierik RLM (1987) In vitro Culture of Higher Plants. Martins Nijhoff Publishers, Dordrecht, the Netherlands, pp. 22-97.

Razdan MK (1992) An Introduction to Plant Tissue Culture. Oxford and IBH Publishing, England, pp. 40-62.

Salunkhe CK, Rao PS, Mhatre M (2004) Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. Pla Cell Rep 17: 56-67.

Sakai A, Kobayashi S, Oiyama I (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Pla Cell Rep 9: 30-33.

Sakai A, Kobayashi S, Oiyama I (1991) Survival by vitrification of nucellar cells of naval orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to -196 °C. J Pla Phy 137: 465-470.

Sakai A, Matsumoto T, Hirai D, Niino T (2000) Newly developed encapsulation-dehydration protocol for plant cryopreservation. CryoLetter 21: 53-62.

Sforza R, Boudon P, Greif C (2003) New mealybug species vectoring grapevine leafroll-associated viruses 1 and 3 (GLRaV-1 and -3). Euro J Pla Patho 109: 975-981.

Sforza R, Greif C (2000) Mealybugs and grapevine leafroll. Phytoma 532: 46-50.

Shahrour WG (2004) Molecular characterization of grapevine viruses in Jordan. Master Thesis. Al-Balqa Applied University, As-Salt, Jordan.

Shatnawi M, Shibli R, Qrunfleh I, Bataeineh K, Obeidat M (2007) *In vitro* propagation and cryopreservation of *Prunus avium* using vitrification and encapsulation dehydration methods. J Food Agri Env 5: 204-208.

Shatnawi MA (2006) Micropropagation and germplasm storage of *Prunus amygdalus* by the vitrification method. Jordan J Agri Sci 2: 222-233.

Shatnawi M, Engelmann F, Frattarelli A, Damiano C (1999) Cryopreservation of apices of in vitro plantlets of almond (*Prunus dulcis* Mill). CryoLetter 20: 13-20.

Shibli RA, Jardat A, Ajlouni M, Aljanabi S, Shatnawi M (1997) Micropropagation in wild pear (*Pyrus syriaca*). Sci Hort 68: 237-242.

StatSoft Inc. (1995) STATISTICA for Windows [Computer Program Manual]. Tulsa, OK, USA.

Towill LE (1990) Cryopreservation of isolated mint shoot tips by vitrification. Pla Cell Rep 9: 178-180.

Wang Q, Gafny R, Sahar N, Sela I, Mawassi M, Tanne E, Perl A (2002) Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. Pla Sci 162: 551-558.

Woodrum R, Antcliff A, Krake L, Taylor R (1984) Yield differences between sultana clones related to virus status and genetic factors. Vitis 23: 73-83.