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Meristem tip culture for i*n vitro* eradication of grapevine leaf roll-associated virus-1 (GLRaV-1) and grapevine fan leaf virus (GFLV) from infected flame seedless grapevine plantlets

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Resumen

Grapevine (*Vitis vinifera* cv. Flame Seedless) was found infected with viral diseases; showing thicker leaves than normal, brittle, with margins rolled downwards and yellowish, which were identified as Grapevine Leaf roll-associated Virus-1 (GLRaV-1). Other symptoms were observed on leaves. The infected leaves showed malformation with abnormal gather primary veins, giving the leaf the appearance of an open fan, including yellowing and mosaic pattern on leaves, or bright yellow bands along major veins. Fan-shaped leaves were associated with mosaic or vein banding symptoms, these observations were identified as Grapevine Fan leaf Virus (GFLV). Both viruses were detected using Double Antibody Sandwich – Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The use of tissue culture was investigated as a mean to eliminate the two viruses. Virus-free plants were produced within six months using meristem tip culture. Woody plant (WP) medium supplied with benzyl amino purine BAP (4.44 µM) was used for shoot proliferation, and indole butyric acid IBA (0.2 µM) for plantlets rooting. Before acclimatization, the plantlets were submitted to DAS-ELISA and RT-PCR in order to evaluate virus eradication. GLRaV-1 and GFLV free plants (95 and 90 %, respectively) were obtained from the optimum size (0.5 mm) of meristem tips (as indexed by DAS-ELISA). Of these, 82.5 and 75 % plants were found negative for GLRaV-1 and GFLV, respectively, as diagnosed by RT-PCR. RT-PCR and meristem tip culture were found to be reliable methods for virus indexing and elimination of GLRaV-1 and GFLV.

Keywords: Grapevine, meristem tip culture, eradication, DAS-ELISA, RT-PCR.

INTRODUCTION

Grapevine is economically one of the most important cultivated fruit species in the world, mostly because of the wine industry but also due to the demand for fresh and dried fruits (Vivier & Pretorius, 2002, *Trends Biotechnol* 20: 472-78). In Egypt, grapevine is the second fruit crop which plays an important role in the agricultural economy. The annual production is about 1.1 million tones of table grapes and dried fruits. The total area for grape production was approximately 65000 ha in 2005 (FAO, 2005, *Annual report* http://www.FAO.org/ag/ar).

Many plants are internally infected with viruses, which cause less vigorous

growth, necrosis, curling of leaves, streaks in leaves or flowers, yield decrease and plant death (Quak, 1977, *Applied and fundamental aspects of plant cell tissue and organ culture, J Reinert and Y P S Bajaj Eds. Springer Verlag Academic press, New York*). Grapevine fan leaf virus (GFLV), Grapevine fleck virus (GFKV), Grapevine virus A (GVA), Grapevine leaf roll associated virus-1 (GLRaV-1), Grapevine leaf roll associated virus-3 (GLRaV-3), Tomato ring spot virus (ToRSV) and Peach rosette mosaic virus (PeRMV) were found to be widely spread (by different degrees) in grapevine nurseries and are considered as an important grapevine viruses in Egypt (Shalaby et al., 2007, *J Agric Sci Mansoura Univ* 32(2): 755-63).

Viruses and other pathogens elimination from planting material is important because most fruit trees are produced by vegetative propagation. If present, disease agents will be readily perpetuated, albeit unwittingly, in the progeny. Moreover, once infected plants are established in commercial orchards or vineyards, they are not amenable to any curative or therapeutic control measures (Rowhani et al., 2005, *Annu Rev Phytopathol* 43: 261-78). Chemotherapy directed to plant viral diseases has evolved significantly. Not only substances that inhibit viral replication, but also induce resistance, have been discovered. The most frequently studied compounds are antimetabolites, substances capable of blocking the virus nucleic acid synthesis (Vicente and De Fazio, 1987, *Fitopatologia Brasileira* 12: 21-26). These compounds, both natural and synthetic, express an antiviral effect, but none of them present a satisfactory selective action that would enable them to be used in specific prophylaxis and in large-scale therapy of plant viral diseases (Hansen & Lane, 1985, *Plant Disease* 69: 134-35). Thus, there is no chemical treatment to eradicate plants of virus infection; however, viruses are not generally spread to the progeny through seed. Two methods to free vegetative propagated plants of viruses are meristem tip culture and thermotherapy (Pio-Ribeiro et al., 1993, *Fitopatologia Brasileira* 18: 265; Nascimento et al., 2003, *Scientia Agricola* 60(3): 525-30).

In vitro meristem tip culture is an efficient method for obtaining virus free material from a wide range of plants. Morel and Martin (1952) (*European Journal of Scientific Research* 18: 155-64) did the pioneering work on the establishment of virus-free dahlias using apical meristem culture. Nowadays, many important horticultural crops (as grapevine) are routinely freed of viral contamination using this procedure depending on the fact that the shoot apical meristem and first set of primordial leaves in an elongating shoot are generally not connected to the vascular system of the plant and therefore, they are not contaminated by viruses that travel through the vascular system (Gomes et al., 2004, *Acta Horticulture* 652: 425-32).

The objective of this study was the detection of GLRaV-1 and GFLV virus diseases in grapevine cv. Flame Seedless by DAS-ELISA and RT-PCR, and in vitro production of virus-free plantlets by meristem tip culture.

MATERIALS AND METHODS

This study was carried out in the Virus and phytoplasma Research Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. The preliminary and the main experiments were done to: (i) detect and eliminate GLRaV-1 and GFLV from infected explants, and (ii) propagate grapevine cultivar "Flame Seedless" using meristem tip culture.

Collection and maintenance of viral cultures

Grapevine cv. Flame Seedless showed many symptoms such as thick leaves, brittle, with margins rolled downwards, yellowish, yellow mosaic pattern on leaves or bright yellow bands along major veins. Fan-shaped leaves with mosaic or vein

banding symptoms were collected. Meristem tips with adjacent primordial leaves were taken as explants for further detection and in vitro culture.

Virus detection of naturally infected and in vitro grown plants

To establish a virus-free plant the apical dome plus two to four subjacent primordial leaves were carefully excised, to avoid the contamination with infected sap from more mature leaves or stem tissues, and then were placed in suitable culture medium. To enhance the survival rate, meristem tips were chosen at the stage of rapid growth.

DAS-ELISA

Double Antibody Sandwich-Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) (Clark & Adams, 1977, Journal of General Virology 34: 475-83 and modified by Bosica et al., 1991, Vitis 30: 97-05 and 1995, Vitis 34: 171-75) was carried out by coating Nunc Polysorp immunoplates with 200 µl per well of antibody solution in carbonate buffer. The samples were tested for GLRaV-1 and GFLV. Commercial kits produced by Agritest, Valenzano, Italy were used. Alkaline phosphatase (AP) conjugated was added according to manufacturer instructions. Plates were read at 405 nm at 30 min intervals for 2 hrs in a BioTex-Elx808 automatic reader (Bio Tex, Highland Park, Winooski, VT, USA), zeroed with an empty plate. Controls were included systematically and each sample was loaded in two different wells. Samples were considered positive when the mean absorbance was at least three standard deviation units above the negative control.

RNA Extraction of GLRaV-1 and GFLV, and RT-PCR

Viral RNA was extracted from infected young leaves collected from grapevine cv. Flame Seedless samples (both under field and in vitro conditions) using RNeasy Plant Mini Kit (Qiagene, Inc) according to manufacturer's instructions. RT-PCR was done using QIAGEN One Step RT-PCR Enzyme Mix (Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase and HotStartTaq™ DNA Polymerase) according to manufactories instructions and performed in thermocycler (Apollo Instrumentation, model ATC 401). Primer pairs for GLRaV-1 and GFLV resulted in an amplification products of 275 and 1557 bp (Kristina et al., 2000, Journal of Virological Methods 86: 101-06; Pejman et al., 2001, Journal of General Virology 82: 1791-95, respectively). The PCR product was analyzed on 1 % agarose gel at 120 volt. Agarose gel stained with ethidium bromide 10 µg/ml (Sambrook et al., 1989, Molecular cloning: A laboratory manual (2ed.), Cold Spring Harbor Laboratory, New York), visualized by examination under a UV transilluminator and photographed using Gel Documentation System (Alpha Innotech Corporation). The 1 kb DNA ladder molecular weight marker (GiBcoBRL, Inc.) was used to determine the size of RT-PCR products.

Plant material for multiplication experiment

Actively growing shoots of 5 cm length were cut from 10 – 15 days old vegetative shoots of an own-rooted one and half-year-old grape transplants (*Vitis vinifera* L.). Transplants of Flame seedless grape cultivar grown in the experimental insect proof glasshouse were used in this study. Shoots were collected during the period of active growth (April to May) to initiate shoot cultures, when lower phenolic level in tissues is associated with higher survival percentage during these months. They were put into plastic bags and taken to the laboratory immediately.

Explants sterilization

Firstly, shoots were stripped of leaves then rinsed thoroughly under running tap water for 10 min, after that the terminal shoot tips (5-10 mm long) containing the apical meristem and few leaves primordia were excised by a sharp blade, then transferred to the culture cabinet (Laminar airflow hood) followed by surface

disinfected by immersion in 70 % ethanol for 10 sec. and soaked for 20 min in 1.05 % (v\v) sodium hypochlorite solution NaOCl (20 % Clorox® as a commercial bleach) and with the addition of two drops of Tween-20 (0.1 %) as a surfactant agent to enhance spreading. They were followed by three rinses, five minutes each, in sterilized distilled water to remove all traces of Clorox. Finally, sterilized shoot meristems with 2-4 leaves primordia were used as experimental materials.

Culture conditions

Grape shoot-tips (5–10 mm) were micropropagated on a basal culture medium contained full-strength inorganic salts of woody plant medium WP (Lloyd and McCown, 1980, International Plant Propagators Society proceedings 30: 421-27), in addition to 3 % (w\v) sucrose, and solidified with 0.8 % (w\v) agar (Bacto agar – DIFCO Laboratories). The pH of every medium was adjusted to 5.7±0.05 with 0.1 N NaOH or HCl prior to addition of agar. The medium was poured into the culture jars, each contained 25 ml of the medium prior to autoclaving. After capping with autoclavable polypropylene lids, medium was autoclaved at 121˚C and 1.2 kg cm^{-2} for 20 min.

Culture establishment

Explants were placed in culture vessels on a free plant growth regulators WP medium. These explants were incubated at 25±2˚C under illuminated conditions (16-h photoperiod with light provided by 40-watt, cool-white, fluorescent lamps). After two weeks, uncontaminated shoot tips were divided into meristem tips without leaves primordia (0.5 mm) and meristem tips with 2 leaves primordia (1 mm), then transferred to WP medium without cytokinin (control plantlets) or on WP-media supplemented with varying concentrations of the cytokinin type benzyl amino purine (BAP) (Sigma chemical Co. Ltd., St. Louis, MO), as multiplication media. In all cytokinin treatments, WP media were supplemented with 0.2 µM IBA as an auxin. After two weeks, the explants gave rise to greening shoots. The rapidly growing shoots were divided and sub cultured on fresh medium every 4 weeks under similar conditions.

Effect of different concentrations of cytokinin on shoot proliferation

This experiment included different treatments of cytokinin. Multiplication of meristem tips was investigated as affected by different concentrations of cytokinin BAP at 4 concentrations (0.00 – 2.22 – 4.44 and 6.66 µM BAP). Each concentration was repeated three times (three replications) and twenty culture shoots were used per replication, i.e. every treatment consisted of 60 jars with one explant per jar. To evaluate the in vitro growth in media supplemented with the cytokinin and their concentrations, multiplication data were determined on the following parameters: total number of shoots (longer than 5 mm) produced from each explant, the mean length of the shoots per culture, leaves number per explant, roots number per explant and the mean length of the roots per plantlet after three multiplication cycles each of 4 weeks.

Statistical analysis

Randomized complete design of two experimental factors: factor A (meristem tip length) and factor B (cytokinin concentration) was used. ANOVA was used to analyze the influence of treatments on grape shoots proliferation. The mean comparisons were made using Duncan's multiple range test at 1 % significant level (Duncan, 1955, Biometrics 11:1-42).

RESULTS

Many factors influence the grapevine in vitro approach. The factors considered in this study were the effect of cytokinin concentration and explant size on shoot multiplication of Flame Seedless grapevine cultivar. Cytokinin application in subculture stage resulted in considerable shoot proliferation; therefore significant differences occurred among explants as shown in tables (1 to 5).

The effect of cytokinin concentration and explant size. Most meristem tips cultured in WP medium amended with BAP and IBA sprouted within 2 weeks of culture and shoot differentiation occurred. The shoots were further multiplied in the same medium. Significant differences were occurred in the number of axillary shoots in response to different cytokinin concentrations. About 1–13.1 shoots appeared from a single cultured meristem. The significant higher shoots numbers were obtained in the fan leaf virus-infected plantlets with 6.66 and 4.44 µM (13.1 and 12.2 shoots) compared to 6.8 and 1 shoots for the decreased concentrations (2.22 µM and the control), respectively (Table, 1) with significant differences between them. Meantime, significant differences were observed between 0.5 and 1 mm explant size for axillary shoots number per explant (Table, 1). The higher average shoots numbers were 13.1 and 12.8 which obtained with the highest BAP concentration. On the other hand, callus was also formed near the base of the meristem and then a number of small shoots appeared according to the BAP level.

Table 1. The effect of explant size and cytokinin concentration on the shoots number of the virus-infected plantlets from Flame Seedless grapevine cultivar.

Shoots number						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GFLV-infected plantlets	0.5	0.8	3.7	5.8	5.3	3.90
	1	1.0	6.8	12.2	13.1	8.28
	Mean B	0.9	5.25	9.0	9.2	
LSD A(0.01) = 1.016, LSD B (0.01) = 1.442, LSD A×B (0.01) = 1.908						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GLRaV-infected plantlets	0.5	0.9	3.9	5.8	7.3	4.48
	1	1.0	6.2	10.1	12.8	7.53
	Mean B	0.95	5.05	7.95	10.05	
LSD A(0.01) = 0.635, LSD B (0.01) = 0.828, LSD A×B (0.01) = 1.313						

Data mean separation by Duncan's multiple range test at the 1 % level.
Data were taken as the average of 3 subcultures. A (Factor): Explant size (mm).
B (Factor): Cytokinin concentration (µM).

At the same time, the higher concentrations (6.66 and 4.44 µM) resulted in a significant reduction in shoot length. The significantly higher shoot lengths (11.2 and 10.7 cm) were obtained by the low concentration (2.22 µM) in comparison with 4.6 and 6.7 cm shoot length with the highest tested concentration (6.66 µM) (Table, 2). The difference between the two tested explant size was significant.

On the other hand, leaves number per explant were not strongly affected by successive increase in BAP concentration. However, BAP at all tested concentrations resulted in significant effect compared to control. It is worth to indicate that most values of BAP concentrations were significantly equal. Thus, there were no

significant differences between the diverse concentrations of BAP, only with respect to leaves number (Table, 3). The difference between 0.5 and 1 mm explant size was only significant at the lowest BAP level (2.22 µM).

Table 2. The effect of explant size and cytokinin concentration on the shoots length of the virus-infected plantlets from Flame Seedless grapevine cultivar.

Shoots length (cm)						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GFLV-infected plantlets	0.5	6.9	8.4	6.9	6.7	7.23
	1	8.3	11.2	7.6	7.3	8.60
	Mean B	7.6	9.8	7.25	7.0	
LSD A(0.01) = 0.825, LSD B (0.01) = 1.075, LSD A×B (0.01) = 1.872						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GLRaV-infected plantlets	0.5	5.2	6.8	6.1	4.6	5.68
	1	8.4	10.7	7.3	5.8	8.05
	Mean B	6.8	8.75	6.7	5.2	
LSD A(0.01) = 0.645, LSD B (0.01) = 0.842, LSD A×B (0.01) = 1.32						

Data mean separation by Duncan's multiple range test at the 1 % level.
Data were taken as the average of 3 subcultures. A (Factor): Explant size (mm).
B (Factor): Cytokinin concentration (µM).

As illustrated in Table (4) the most axillary roots number per explant was stimulated by lowest concentration of BAP (3.2 and 2.8 roots respectively) followed by higher concentrations (4.44 and 6.66 µM). Also, the difference between 0.5 and 1 mm explant size was only significant at the lowest BAP level (2.22 µM).

Table 3. The effect of explant size and cytokinin concentration on the leaves number of the virus-infected plantlets from Flame Seedless grapevine cultivar.

Leaves number / explant						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GFLV-infected plantlets	0.5	6.4	8.9	9.3	9.8	8.60
	1	8.5	11.6	10.4	10.9	10.35
	Mean B	7.45	10.25	9.85	10.35	
LSD $A_{(0.01)}$ = 1.031, LSD $B_{(0.01)}$ = 1.532, LSD $A\times B_{(0.01)}$ = 1.779						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GLRaV-infected plantlets	0.5	5.3	7.7	8.2	7.1	7.08
	1	8.9	11.5	9.6	8.5	9.63
	Mean B	7.1	9.6	8.9	7.8	
LSD $A_{(0.01)}$ = 0.861, LSD $B_{(0.01)}$ = 1.115, LSD $A\times B_{(0.01)}$ = 1.843						

Data mean separation by Duncan's multiple range test at the 1 % level.
Data were taken as the average of 3 subcultures. A (Factor): Explant size (mm).
B (Factor): Cytokinin concentration (µM).

Table 4. The effect of explant size and cytokinin concentration on the roots number of the virus-infected plantlets from Flame Seedless grapevine cultivar.

Roots number / explant						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GFLV-infected plantlets	0.5	0.0	1.7	2.3	1.4	1.35
	1	0.0	3.2	2.5	1.1	1.70
	Mean B	0.0	2.45	2.4	1.25	
LSD A $_{(0.01)}$ = 0.408, LSD B $_{(0.01)}$ = 0.561, LSD A×B $_{(0.01)}$ = 0.623						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GLRaV-infected plantlets	0.5	0.0	1.3	1.9	1.1	1.08
	1	0.0	2.8	2.2	1.0	1.50
	Mean B	0.0	2.05	2.05	1.05	
LSD A $_{(0.01)}$ = 0.402, LSD B $_{(0.01)}$ = 0.504, LSD A×B $_{(0.01)}$ = 0.794						

Data mean separation by Duncan's multiple range test at the 1 % level.
Data were taken as the average of 3 subcultures. A (Factor): Explant size (mm).
B (Factor): Cytokinin concentration (µM).

Moreover, significant differences were detected for root length as compliance to the change of the cytokinin concentration. 6.66 µM of BAP was the best concentration of cytokinin in regards to root length (13.2 and 11.7 cm) (Table, 5). Also, the difference between the two tested explant sizes was only significant at the highest BAP concentration (6.66 µM).

Generally, the aforementioned data showed that the small size of grape meristem tip (0.5 mm) cultured on WP media in combination with different concentrations of BAP had the least effect on most growth characters of the plantlets grown in vitro than the large size (1 mm).

Table 5. The effect of explant size and cytokinin concentration on the roots length of the virus-infected plantlets from Flame Seedless grapevine cultivar.

Roots length (cm)						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GFLV-infected plantlets	0.5	0.0	7.2	5.9	9.7	5.70
	1	0.0	5.3	6.4	13.2	6.23
	Mean B	0.0	6.25	6.15	11.45	
LSD A $_{(0.01)}$ = 0.983, LSD B $_{(0.01)}$ = 1.186, LSD A×B $_{(0.01)}$ = 1.925						
	Explant size (mm)	Cytokinin concentration (µM) 0.00	2.22	4.44	6.66	Mean A
GLRaV-infected plantlets	0.5	0.0	6.9	5.2	8.4	5.13
	1	0.0	6.3	5.7	11.7	5.93
	Mean B	0.0	6.6	5.45	10.05	
LSD A $_{(0.01)}$ = 0.975, LSD B $_{(0.01)}$ = 1.098, LSD A×B $_{(0.01)}$ = 2.186						

Data mean separation by Duncan's multiple range test at the 1 % level.
Data were taken as the average of 3 subcultures. A (Factor): Explant size (mm).
B (Factor): Cytokinin concentration (µM).

Virus detection

Grapevine (*Vitis vinifera* L. cv. Flame seedless) was found infected with viral diseases showing thicker leaves than normal, brittle, with margins rolled downwards and yellowish, which were identified as Grapevine Leaf roll-associated Virus-1 (GLRaV-1). Other symptoms were observed on leaves. Infected leaves showed malformation with abnormal gathered primary veins, giving the leaf appearance of an open fan, including yellowing and mosaic pattern on leaves or bright yellow bands along major veins. Fan-shaped leaves with mosaic or vein banding symptoms were identified as Grapevine Fan leaf Virus (GFLV) (**Fig. A**).

The detection of both viruses was carried out by DAS-ELISA and RT-PCR. Virus indexing by RT-PCR was found to be a reliable method in comparison to DAS-ELISA because the virus infection was detected in some plants which were found to be negative by DAS-ELISA. In RT-PCR, the expected amplification of 275 bp and 1557 bp were obtained in virus infected plants (naturally and in vitro grown) with GLRaV-1 and GFLV respectively, while no amplification was obtained in GLRaV-1 and GFLV-free plants (**Fig. B**).

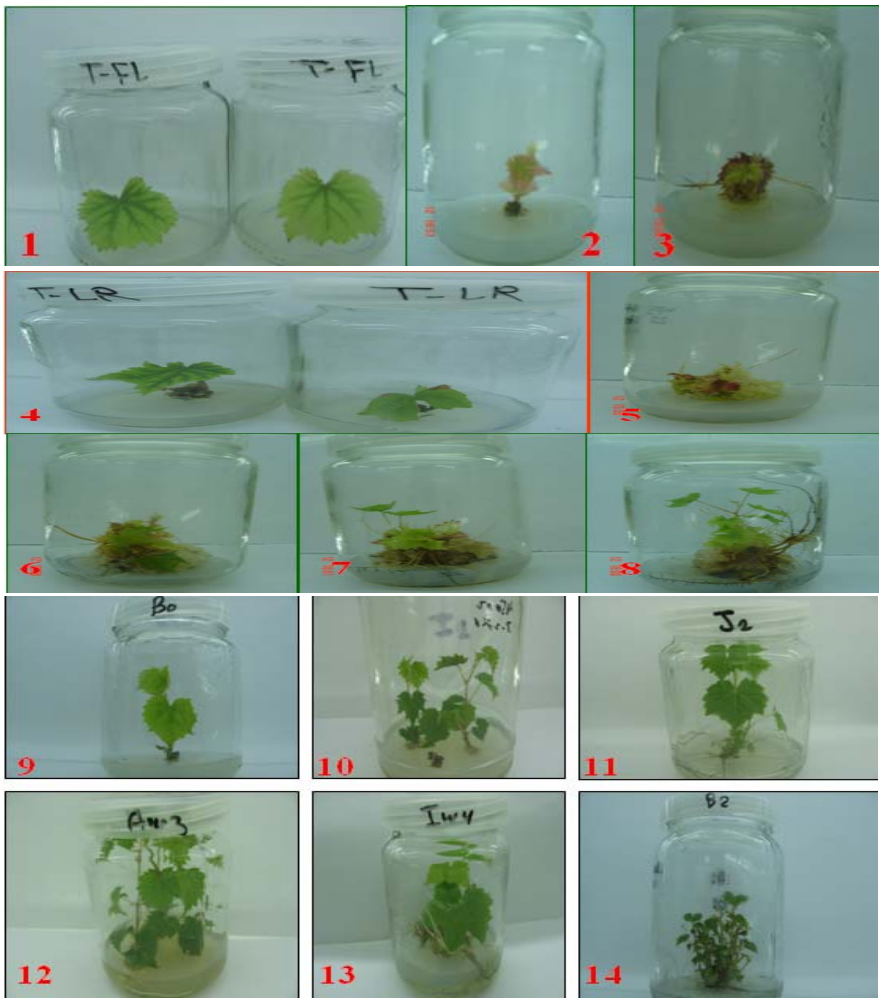

Figure A. Successive stages of Grapevine Fan Leaf viral infection (1, 2 and 3). Grapevine Leaf Roll viral symptoms (4, 5, 6, 7 and 8). Healthy plantlets after the elimination of the two viruses (9, 10, 11, 12 and 13). BAP effect on multiplication rate (14).

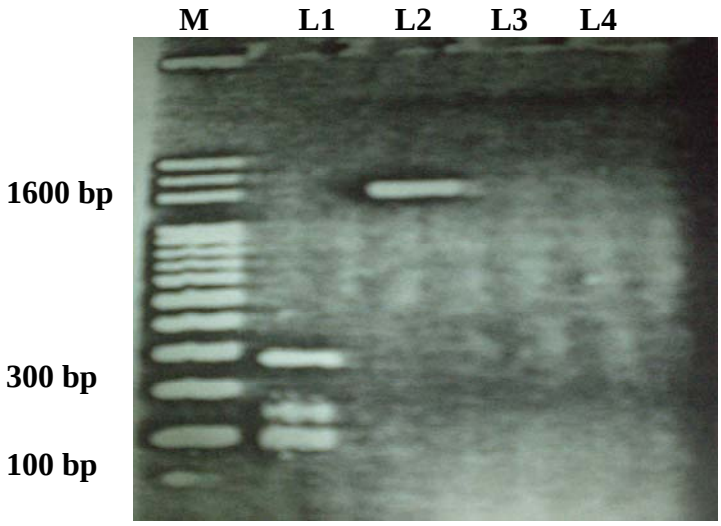

Figure B. Agarose gel electrophoresis analysis of RT-PCR products obtained from total RNA extracted from grapevine tissues individually infected with GLRaV-1 (lane 1) or GFLV (lane 2). The virus specific amplified bands corresponding to 275 bp for GLRaV-1 and 1557 bp for GFLV. Lane M, 100 bp ladder marker.

Effect of meristem size on its establishment and virus elimination

The small size meristem tips (0.5 mm) which were taken in this study, transformed both in callus and shoots. They were tested for the presence of the two viruses individually. While 1.0 mm long meristem tips produced shoots only. Meristem tips of 0.5 mm size were found to be optimum for GFLV and GLRaV-1 elimination from infected grapevines. Large sized meristem 1.0 mm carried the virus particles with it and many of shoots produced from them were found to be virus infected (Table, 6). Virus-free plants (90 and 95 %) were obtained from the optimum size (0.5 mm) of meristem tips (as indexed by DAS-ELISA). From which 75 and 82.5 % plantlets were found negative for GFLV and GLRaV-1 respectively, as indexed by RT-PCR. On the contrary, virus-free plantlets decreased when the large size of explants (1 mm) were used, to obtain 55-60 % of the cultured plantlets virus-free of GFLV and GLRaV-1 respectively, as indexed by RT-PCR.

Table 6. The effect of the meristem tip size on the production of fan leaf and leaf roll viruses-free plantlets of Flame Seedless grapevine cultivar.

Virus type	Meristem size mm	Tissue differentiation	No. of shoots grown *in vitro*	Virus indexing			
				DAS-ELISA		RT-PCR	
				Virus–free plants	Virus-free plants (%)	Virus–free plants	Virus-free plants (%)
GFLV	0.5	Callus+shoots	40	36	90	30	75
	1.0	Shoots	40	29	72.5	22	55
GLRaV -1	0.5	Callus+shoots	40	38	95	33	82.5
	1.0	Shoots	40	31	77.5	24	60

DISCUSSION

Tissue culture has recently become an accepted profitable and established technique for propagation of many vegetatively propagated plants (Nasr El-Din et al., 1997, *Bull Fac Agric Cairo Univ* 48: 129-42). Moreover, micropropagation could provide a mean to clone superior selections rapidly from conventional breeding programs, or provide the basis for in vitro genetic manipulation or selection (Libby and Ahuja, 1993, *Micropropagation of Woody plants*, *Kluwer Academic Publishers, Dordrecht, the Netherlands*).

In the present study, shoot formation from the shoot tip meristem was achieved in all the treatments. Two factors were considered in this study: (i) explant size and (ii) cytokinin concentration. Both factors revealed significant role about the ability of shoot tip explants for axillary shoot proliferation. The present results proved that cytokinin requirement is an important consideration for in vitro culture of grapevine cultivars as previously reported by Wanas et al., (1999) (*Arab Univ Agric Sci* 7(1): 179-90). However, the efficiency of each concentration varied among the explant size. Similarly, Robert and Loyd (1975) (*J Amer Soc Hort Sci* 100(2): 200-02) recorded that cytokinin may possess unique nutritional requirements, for Concord cv. which exhibit suitable axillary shoot proliferation on MS medium. Experiments with benzylaminopurine (BAP) concentrations show that there is a very narrow concentration peak for maximum shoot production of 'Remaily Seedless' grapevine cultivar. Optimal axillary shoot proliferation with adequate shoot elongation rates occurred when 5 µM BAP used into MS medium (Raymond et al., 1984, *New York's Food and Life Sciences Bulletin* 109: 1-9; Han et al., 2003, *Vitis* 42(3): 163-64); There was a tendency for more than one bud to initiate growth on BAP containing media, they added that the limited degree of shoot micropropagation in the control media may be due to the presence of a small endogenous cytokinin content in the explants. Under the conditions of this study, the shoots number of all treatments appeared to increase with increasing BAP tested concentrations, and one can observe that they produced maximal shoot number at the maximal tested concentration. Such effect of BAP concentration is coming back to the cytokinin dual role: Firstly, cytokinin eliminates the apical dominance phenomenon. Secondly, cytokinin encourages rapid cellular division (Kadota and Niimi, 2003, *Plant Cell Tissue and Organ Culture* 72: 261-65). On the other hand, leaves number appeared to increase with increasing BAP test concentrations, and the cause probably returning to the reduction of interval between nodules (Dzazio et al., 2002, *Revista Brasileira de Fruticultura* 24: 3, 759-64). Nevertheless, the shoot length is not regulated by the same principle, values of shoots lengths appeared to decrease with increasing BAP test concentrations, and the reason may be due to the antagonism relation between high number of shoots and their lengths.

There are various explanations for virus elimination during in vitro culture e.g. action of growth regulators particularly cytokinin (Barlass and Skene, 1982, *Scientia Hort* 17: 333-41), phenol-amines (Martino-Tanguy, 1985, *Plant Growth Regul* 3: 381-99), loss of enzymes necessary for viral replication, and viral RNA degradation due to cell injury during explant excision. Virus elimination depends on different factors such as meristem size, physiological conditions of mother plants, and meristem position on it. The larger the size of the meristem cultured, the greater is the number of regenerated plants, where the number of virus-free plantlets obtainable is inversely proportional to the size of the cultured tips (Faccioli and Marani, 1998, *Plant virus disease control*, *A. Hadidi; R K Khetarpal and H Koganezawa Eds*, *APS Press Publishers St Pau, Minesota*). In our experiment, as the size of meristem decreases, the percentage of obtaining the virus-free plants increases for the two studied viruses, and this may be resulted to the fact that the meristem tips in an elongating shoots are generally not connected to the vascular

system of the plant and therefore they are not contaminated by viruses that travel through the vascular system.

CONCLUSION

Viral eradication using meristem tip culture technique is considered one of the most reliable methods for obtaining virus-free stocks from propagative material that comes from infected plants, aided or not by thermo- and/or chemotherapies. These methodologies allow quick propagation of plant material, producing healthy plants from a single individual in a short period of time, regardless of location or season of the year. RT-PCR was found to be more sensative method in comparison to biological indexing and DAS-ELISA. Finally, a virus cleaning programme was therefore set up in order to eliminate both GLRaV-1 and GFLV from the commercial varieties and rootstocks using in vitro culture technique. This would provide us with virus-free material in order to carry out transmission studies, maintain the existing of grapevine virus-free materials, and to avoid the introduction of new virus strains from imported varieties. This will help to minimize virus infection and hence produce better quality of grape (*Vitis vinifera* L.) plants.

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