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Elimination of Grapevine Fan leaf Virus (GFLV) and rapevine Leaf Roll-associated Virus-1 (GLRaV-1) from infected grapevine plants using meristem tip culture

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Grapevine (*Vitis vinifera* L. cv. Thompson seedless) was found to be infected with viral diseases showing thicker leaves than normal, brittle, with margins rolled downwards and yellowish, which identified as *Grapevine leaf roll-associated virus-1* (GLRaV-1). Other symptoms observed on infected leaves were, malformation with abnormal gathered primary veins, giving the leaf the appearance of an open fan, including yellowing and mosaic pattern or bright yellow bands along major veins. Fan-shaped leaves may or may not be present with mosaic or vein banding symptoms which identified as *Grapevine fanleaf virus (GFLV)*. Detection of the viruses was carried out by double antibody sandwich-enzyme-linked immunosorbent 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 medium supplied with benzylamino purine (BAP) (1.5 mg/L) for shoot proliferation, and Indol butyric acid (IBA) (0.05 mg/L) for plants rooting. Before acclimatization, the plantlets were submitted to DAS-ELISA and RT-PCR in order to evaluate virus eradication. GFLV- and GLRaV-1-free plants (92.5 and 95 %, respectively) were obtained from the optimum size (1 mm) of meristem tips (as indexed by DAS-ELISA). Of these, 85 and 87.5 % plants were found negative from GFLV and GLRaV-1, respectively, as indexed by RT-PCR. Virus indexing by RT-PCR was found to be a reliable method, thus proving the efficiency of this method (meristem tip culture) for GLRaV-1 and GFLV elimination.

Key words: Grapevine, Meristem tip culture, Elimination, DAS-ELIS and 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 fruit **(Vivier and Pretorius, 2002).** In Egypt, Grapevine is of great importance and plays an important role in the agricultural economy; the annual production is 1.1 million tones of mainly table grapes and dried fruit. The total area of grape production was approximately 65000 ha in 2005. Many plants are internally infected with viruses, and these resulted in less vigorous growth, necrosis, curling of leaves, streaks in leaves or flowers, decreases in yield, and plant death **(Quak, 1977).**

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Grapevine fane leaf virus (GFLV), Grapevine fleck virus (GFKV), Grapevine virus A (GVA),Grapevine leafroll associated virus -1 (GLRaV-1), Grapevine leafroll 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 propagation material of 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. 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).** 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 and Lane, 1985).** Freedom from viruses and other pathogens in deciduous planting grapevine and are considered as economically important grapevine viruses in Egypt **(Shalaby *et al.*, 2007).** There is no chemical treatment to eliminate a plant of virus infection; however, viruses are not generally spread to the progeny through seed. Two methods to free vegetatively propagated plants of virus are meristem tip culture and thermotherapy.

stocks is important because nearly all plants for plantings are produced by vegetative propagation. If present, disease agents will be readily perpetuated, albeit unwittingly, in the progeny. Moreover, once diseased plants are established in commercial orchards or vineyards, they are not amenable to any curative or therapeutic control measures **(Rowhani *et al.*, 2005).** Generally, ***in vitro*** culture is a useful tool for rapid propagation of grapevines and the *in vitro* meristem tip culture is an efficient method for obtaining virus-free plants from a wide range of plants that comes from infected sources, aided or not by thermo-and /or chemotherapies **(Mellor and Stace-Smith, 1970).** Many important horticultural crops are routinely freed of viral contamination using this procedure **(Gomes *et al.*, 2004).** A virus-free plant can be established if the explant is carefully excised so

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as not to contaminate it with sap from more mature leaves or stem tissues and is placed in suitable culture medium. In this study we reported the detection of GLRaV-1 and GFLV in grapevine (*Vitis vinifera* L. cv. Thompson seedless) by DAS-ELISA and RT-PCR, and *in vitro* production of virus-tested grapevine by meristem tip culture.

MATERIALS AND METHODS

The preliminary and the main experiments were done during 2006 to detect and eliminate GLRaV-1, GFLV from infected explants by using meristem tips culture and study the rapid propagation of grapevine cultivar "Thompson seedless" by shoot tips culture.

1. Collection and maintenance of viral cultures

Grapevine cv. Thompson seedless showing characteristic symptoms on leaves including thicker leaves than normal, brittle, with margins rolled downwards, yellowish, yellow mosaic pattern on leaves or bright yellow bands along major veins. Fan-shaped leaves may or may not be present with mosaic or vein banding symptoms on leaves were collected. Young leaves and meristem tips were chosen for further detection and to produce virus-free plants.

2. Virus detection of naturally infected and *in vitro* grown plants:

2.1. Double antibody sandwich - Enzyme linked immunosorbent assay (DAS- ELISA):

Double antibody sandwich - Enzyme linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977 and modified by Bosica *et al.*, (1991) and (1995)) was carried out by coating Polysorp immunoplates, Nunc with 200 µl per well of antibody solution in carbonate buffer. The samples were tested against GFLV, GLRaV-1. Commercial kits produced by Agritest, Valenzano, Italy were used. Alkaline phosphatase conjugated (AP) were added according to manufacturer instructions. Plates were read at 405 nm at 30 min intervals for 2 hr in a BioTex-Elx808, BioTex, Highland Park, Winooski, VT, USA automatic reader 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.

2.2. RNA Extraction GVLV and GLRaV-I and RT-PCR:

Viral RNA were extracted from infected young leaves collected from grapevine *cv.* Thompson seedless (both under field condition and *in vitro* grown) using Rneasy Plant Minikit (QIAGEN, Inc) according to manufacturer's instructions. RT-PCR was done using QIAGEN OneStep 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 GLRaVI and GFLV were used that resulted in an amplification products of 275 bp and 1557 as reported earlier by Kristina *et al.*, 2000 and Pejman et al., 2001 respectively. The PCR product was analyzed on 1% Agarose gel at120 volt. Agarose gel was stained with ethidium bromide 10 µg / ml (Sambrook *et al.*, 1989), visualized by examination under a UV transilluminator and photographed using Gel Documentation System (AlphaInnotech Corporation). The 1Kb DNA Ladder molecular weight marker (GiBcoBRL, Inc.) was used to determine the size of RT-PCR products.

3. Multiplication experiment :

3.1. Plant material:

Actively growing shoots of 5 cm 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 Thompson seedless grape cultivar grown in the experimental insect proof glasshouse were used in this study. Shoots were collected during a 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.

3.2. 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

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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. At last, sterilized shoot meristems with 2 - 4 leaves primordia were used as experimental materials.

3. 3. 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 medium, 1980), 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 ± 0.1 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.

3.4. Culture establishment:

Explants were placed within 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 (1 mm) and meristem tips with 2 leaves primordia (3 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 0.05 mg l^{-1} IBA as an auxin. After two weeks, the explants gave rise to greening shoots. The rapidly growing shoots were divided and subcultured to fresh medium every 4 weeks under similar conditions.

3.5. Effect of different concentrations of cytokinin on shoot proliferation:

This experiment included different treatments of cytokinin. Multiplication of meristem tips was

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investigated as affected by different concentrations of cytokinin BAP ($C_{12}H_{11}N_5$) at 4 concentrations (0.00 – 0.50 – 1.00 and 1.50 mg l^{-1}). Molecular weight of benzyl amino purine (BAP) = 225.3; therefore, the tested concentrations equal 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 its 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 / explant, roots number / explant and the mean length of the roots per plantlet after three multiplication cycles each of 4 weeks.

3.6. Statistical analysis:

Randomized complete design for two experimental factors: factor A (meristem tip length) and factor b (cytokinin concentration). 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).

RESULTS

1. Virus detection:

Grapevine (*vitis vinifera* L. cv. Thompson seedless) was found infected with viral diseases showing thicker leaves than normal, brittle, with margins rolled downwards and yellowish, which identified as Grapevine Leaf Roll-associated Virus1 (GLRaV-1). Other leaf symptoms observed were malformation with abnormal gathered primary veins, giving the leaf the appearance of an open fan. These symptoms may include a yellow mosaic pattern or bright yellow bands along major veins. Fan-shaped leaves may or may not be present with mosaic or vein banding symptoms which identified as *Grapevine FanLeaf Virus* (GFLV) (Plate 1,2 and 3). . Detection of the viruses was carried out by DAS-ELISA and RT-PCR. RT-PCR was found to be reliable method in comparison to DAS-ELISA because the virus infection was detected in some plant, which were found to be negative in 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. 1).

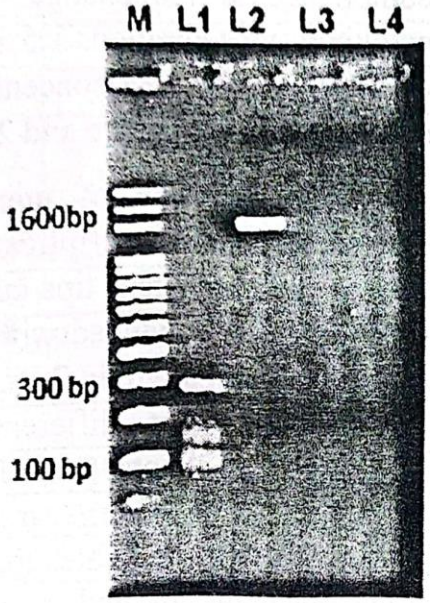

Fig.1. Agarose gel electrophoresis analysis of RT-PCR products obtained from total RNA extracted from grapevine tissues individually infected with (GLRaV-1, lane 1) and Grapevine fan leaf virus (GFLV, lane 2), The virus specific amplified bands corresponding to 275 bp for GLRaV-1 and , 1557 bp for GFLV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

2- Multiplication Experiments:

1. Shoot proliferation:

Many factors influence the grapevine *in vitro* approach. Factors considered in this study were the effect of cytokinin concentration and explant size on shoot multiplication of Thompson seedless grapevine cultivar (Tables. 1 and 2). Cytokinin application in subculture stage resulted in considerable shoot proliferation; therefore significant differences occurred among explants as shown in tables (1 and 2).

2. Cytokinin concentration effect :

Significant differences were occurred in the number of axillary shoots in response to different cytokinin concentrations. The significant higher shoots values were obtained in the fanleaf virus-infected plantlets with 1.5 and 1 mg / l (13.73 and 12.67 shoots with explants 3 mm) compared to 7.3, and 1 shoots for the decreased concentrations (0.50 and 0.00 mg / l), respectively (Table, 1.a) with significant differences between them. In the meantime, significant differences were observed between leaf roll virus-infected plantlets for axillary shoots number per explant as shown in Table (2.a). The highest

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average shoot number was 12.53 which obtained with the highest BAP concentration (1.5 mg / l). At the same time, the higher concentrations (1.50 and 1 mg/ l) resulted in a significant reduction in shoot length. The significantly higher shoot lengths (10.57 and 10.37 cm) were obtained by the low concentration (0.50 mg / l) in comparison with 4.87 and 6.4 cm shoot length with the highest tested concentration (1.50 mg /l) Tables (1.b and 2.b). On the other hand, leaves number were not strongly affected by successive increase in BAP concentration. BAP at all tested concentrations had intermediate effect. It is worth to indicate that most values were significantly equal. Thus, there were no significant differences between the diverse concentrations of BAP, only with respect to leaves number (Table, 1. c.). As illustrated in Tables (1.d. and 2. d.) the most axillary roots number was stimulated by lowest concentration of BAP (3.67 and 3.63 roots respectively) followed by higher concentrations (1 and 1.5 mg / l). Moreover, significant differences were happened for root length as compliance to the change of the cytokinin concentration. 1.5 mg / l of BAP was the best concentration of cytokinin (Tables, 1.e and 2.e).

3.3. Differentiation of meristem culture and shoot multiplication :

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. About 1 – 13.73 shoots appeared from a single cultured meristem. Callus was also formed near the base of the meristem and then a few or many small shoots appeared according to the BAP level (Tables, 1.a and 2.a).

Table 1. The effect of explant size and cytokinin concentration on the *in vitro* growth characters of the fanleaf virus-infected plantlets of Thompson seedless grapevine cultivar.

Table (1. a).

Shoots number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.867	3.867	6.000	5.767	4.125
3	1.000	7.300	12.67	13.73	8.675
Mean B	0.933	5.584	9.335	9.749	6.4
LSD A (0.01) = 1.052, LSD B (0.01) = 1.487, LSD A×B (0.01) = 2.104					

Table (1. b).

Shoots length					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	6.933	8.033	6.800	6.400	7.04
3	7.767	10.57	7.400	7.000	8.18
Mean B	7.35	9.3	7.1	6.7	7.61
LSD A (0.01) = 0.8385, LSD B (0.01) = 1.186, LSD A×B (0.01) = 1.677					

Table (1. c).

Leaves number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	6.667	8.667	9.000	10.00	8.584
3	8.333	11.33	10.33	11.67	10.42
Mean B	7.5	9.999	9.665	10.84	9.5
LSD A (0.01) = 1.160, LSD B (0.01) = 1.640, LSD A×B (0.01) = 2.320					

Table (1. d).

Roots number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.000	1.800	2.533	1.600	1.483
3	0.000	3.667	2.600	1.433	1.925
Mean B	0.000	2.734	2.567	1.517	1.7
LSD A (0.01) = 0.421, LSD B (0.01) = 0.5954, LSD A×B (0.01) = 0.8420					

Table (1. e).

Roots length					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.000	7.333	5.733	8.700	5.442
3	0.000	10.50	13.90	18.40	10.70
Mean B	0.000	8.917	9.817	13.55	8.07
LSD A (0.01) = 0.9406, LSD B (0.01) = 1.330, LSD A×B (0.01) = 1.881					

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 (mg l^{-1}).

Table 2. The effect of explant size and cytokinin concentration on the *in vitro* growth characters of the leaf roll virus-infected plantlets of Thompson seedless grapevine cultivar.

Table (2.a).

Shoots number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.800	3.667	5.400	6.700	4.142
3	1.000	7.333	10.33	12.53	7.798
Mean B	0.9	5.5	7.865	9.615	5.97
LSD A (0.01) = 0.6173, LSD B (0.01) = 0.8730, LSD A×B (0.01) = 1.235					

Table (2.b).

Shoots length					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	6.100	7.033	7.333	4.867	6.333
3	8.633	10.37	8.467	6.033	8.375
Mean B	7.367	8.702	7.9	5.45	7.35
LSD A (0.01) = 0.6149, LSD B (0.01) = 0.8696, LSD A×B (0.01) = 1.230					

Table (2.c).

Leaves number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	6.000	7.667	8.667	7.333	7.417
3	9.333	12.67	10.00	10.33	10.58
Mean B	7.667	10.17	9.334	8.832	9
LSD A (0.01) = 0.8116, LSD B (0.01) = 1.148, LSD A×B (0.01) = 1.623					

Table (2.d).

Roots number					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.000	1.833	1.700	1.267	1.2
3	0.000	3.633	2.167	1.067	1.717
Mean B	0.000	2.733	1.934	1.167	1.46
LSD A (0.01) = 0.3804, LSD B (0.01) = 0.5380, LSD A×B (0.01) = 0.7609					

Table (2.e).

Roots length					
Explant size (mm)	Cytokinin concentration (mg l^{-1})				Mean A
	0.00	0.50	1.00	1.50	
1	0.000	6.267	6.467	8.567	5.325
3	0.000	10.53	15.70	18.80	11.26
Mean B	0.000	8.399	11.08	13.68	8.29
LSD A (0.01) = 0.9151, LSD B (0.01) = 1.294, LSD A×B (0.01) = 1.830					

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 (mg l^{-1}).

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3.4. Effect of meristem size on its establishment and virus elimination :

Small size meristem tips (1 mm) taken in this study transformed in callus and shoots both, and were tested for the two viruses presence or absence each one alone. While 3 mm long meristem tips produced shoots only. Meristem tips of size 1 mm were found to be optimum for elimination GFLV and GLRaV-1 from infected grapevines. Large sized meristem 3 mm carried the virus particles with it and many of shoots produced from them were found to be virus infected (Table, 3). Virus-free plants (92.5 and 95 %) were obtained from the optimum size (1 mm) of meristem tips (as indexed by DAS-ELISA). Of these, 85 and 87.5 % plants were found negative for GFLV and GLRaV-1 respectively, as indexed by RT-PCR.

Table 3. The effect of the meristem tip size in the production of fanleaf and leaf roll viruses-free plantlets of Thompson seedless grapevine cultivar.

Virus type	Size of meristem mm	Tissue differentiation	No. of shoots grown *in vitro*	Virus indexing			
				DAS-ELISA		RT-PCR	
				No. of virus – free plants	% of virus-free plants	No. of virus – free plants	% of virus-free plants
FL	1	Callus + Shoots	40	37	92.5	34	85
	3	Shoots	40	32	80	25	62.5
LR	1	Callus + Shoots	40	38	95	35	87.5
	3	Shoots	40	34	85	22	55

DISCUSSION

Tissue culture has recently become an accepted profitable and established technique for propagation of many vegetatively propagated plants on a commercial basis (**Nasr El-Din *et al.*, 1997**).

Moreover, micropropagation could provide a mean to clone superior selections rapidly from conventional breeding programs, provide the basis for *in vitro* genetic manipulation or selection

(Libby and Ahuja, 1993), and the usage of meristem and shoot-tip cultures for pathogen-free plants as a common practice in the production of virus-free stocks. Two factors were considered in this study, i.e. explant size and cytokinin concentrations. All of these 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 (Wanas *et al.*, 1999). However, the efficiency of each concentration varied among the explant size. Similarly, **Robert and Loyd (1975)** recorded that cytokinin may possess unique nutritional requirements, for Concord cv. which exhibit suitable axillary shoot proliferation on MS medium; Experiments with benzylamino purine (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; and Han *et al.*, 2003); 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 medium 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. Shoots number increasing along with concentration's increasing 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). Nevertheless, the shoot length doesn't submit to 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. On the other hand, leaves number appeared to increase with increasing BAP test

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There are various explanations for virus elimination during *in vitro* culture e.g. action of growth regulators particularly cytokinin **(Barlass and Skene, 1982)**, phenol-amines **(Martino-Tanguy, 1985)**, 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, the virus concerned, physiological condition of mother plants, and meristem of position on it. The larger the size of the meristem cultured, the greater is the number of regenerated plants, which the number of virus-free plantlets obtainable is inversely proportional to the size of the cultured tips **(Faccioli and Marani, 1998)**. In our experiment, as the size of meristem decreases, the percentage of obtaining the virus-free plants increased for the two studied viruses, and this is may be resulted to that the meristem tip in an elongating shoot are generally not connected to the vascular system of the plant and therefore are not contaminated by viruses that travel through the vascular system. RT-PCR was found to be a reliable method in comparison to biological indexing and DAS-ELISA. Using RT-PCR the virus could be detected in some plants, which were found to be negative on 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 by *in vitro* techniques. This would provide us with virus free material in order to carry out yield loss and transmission studies, to maintain the existing of grapevine virus-free materials and to avoid the introduction of new strains from imported varieties. This will help to minimize virus infection and hence produce quality of *vitis vinifera* L. grapevine plants.

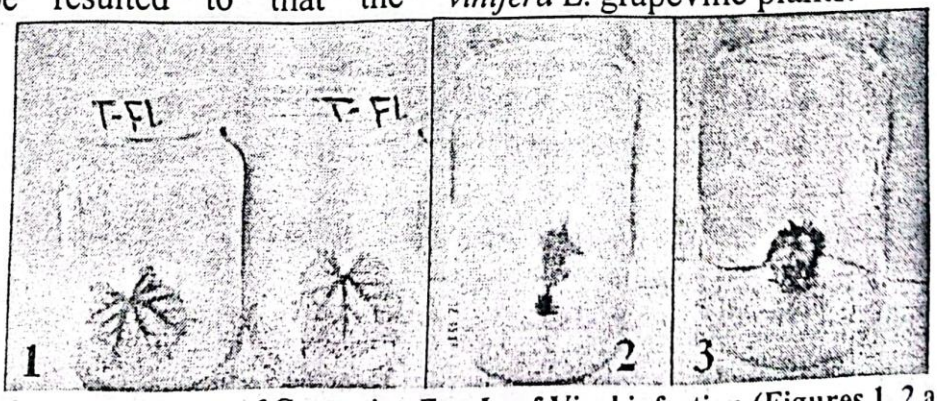

Plate (1): Successive stages of Grapevine Fan Leaf Viral infection (Figures 1, 2 and 3).

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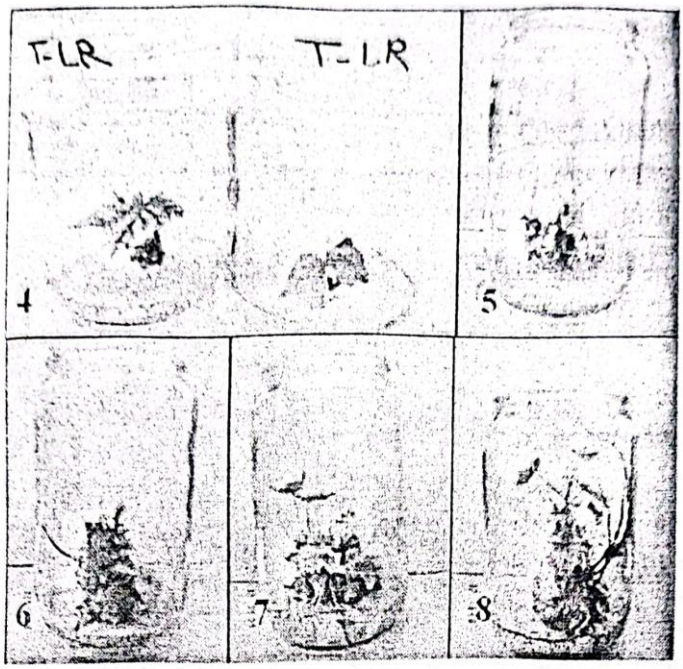

Plate (2): Grapevine leaf roll viral symptoms (Figures 4, 5, 6, 7 and 8).

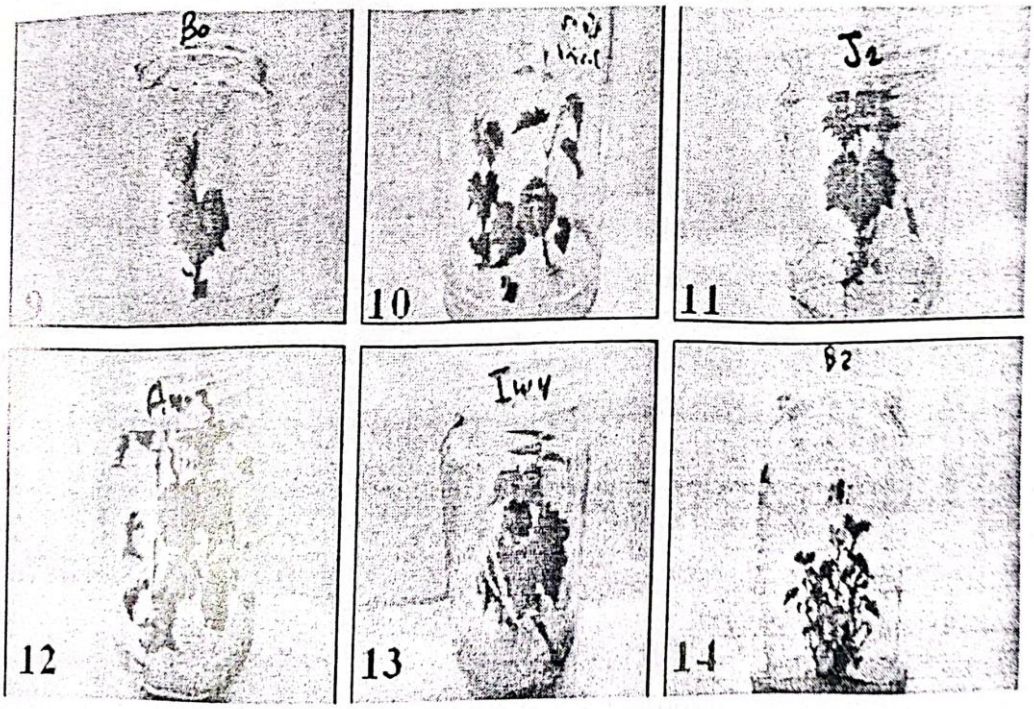

Plate (3): Healthy plantlets after the elimination of the two viruses (Figures 9,10, 11, 12 and 13). BAP effect on multiplication rate (Figure 14).

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