IJANS International Journal of Agricultural and Natural Sciences
Uluslararası Tarım ve Doğa Bilimleri Dergisi
E-ISSN: 2651-3617 1(1): 69-74, 2018

In Vitro Regeneration Of Virus-Free Grapevine (*Vitis Vinifera* L.) İn Some Commercial Cultivars

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Abstract
Commercial cultivars of grapevines (*Vitis vivifera* L.) are very susceptible to virus diseases. The objective of this work was to determine the usefulness of *in vitro* meristem culture to obtain virus-free grapevine plants 5 cultivars of Iranian native grapevine. Cuttings from infected grapevines were rooted in perlite media. Afterward, the apical meristems from the shoots of rooted cuttings were excised (0.1-0.2 mm) and transferred to fresh 1/2MS medium with 0.5 mgL^{-1} of BAP and grown in a culture room until they developed into entire plants. Control plants from infected rooted cuttings and shoot tip culture were assessed for their virus status using RT-PCR mhetod. Results showed control plants derived from cuttings remained virus infected. However, all the meristem-derived plants were virus-free. 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 doubling time was in Fakhri 4, Peykani 4.14, Sultana 4.08, Asgari 4.15 and Shaste Arous 4.11 shoot per 30 days. The used method had high reliable in the propagation and production of disease-free plant. The *in vitro* derived shoots were pretreated with 1 mgL^{-1} IBA, and then directly potted, which caused significant enhancement in root number per shoot; therefore, the time needed for plantlet regeneration was shortened. There was no significant difference between the five cultivars regarding the measured traits.

Keywords: virus-free, grapevine, *in vitro*, meristem

INTRODUCTION

Meristem culture has historically been recognized as perhaps the best means of producing plants free of systemic pathogens. Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops grown in the world today in terms of both total acreage and dollar value and the demand for grape products has increased dramatically[3,4]. In Iran, grape has been vegetatively propagated through hardwood stem cuttings and may carry plant pathogens along with the plant material causing finally reduced vigour and yield of plants [23]. Some diseases are caused by intracellular pathogens of different natures, among which plant viruses are the most important such as fan-leaf virus (GFLV), grape leaf-roll associated viruses (GLRaVs), and grape fleck virus (GFKV) [26, 10, 40]. Using certified materials ensure that the materials have been tasted for known strains of viruses and viruses have been eliminated [27]. So vine plantation must be from certified virus-free sources [43]. One basic drawback of conventional methods, however, is that they don't allow a rapid buildup of grape material that is in limited supply [42]. Thermotherapy and meristem culture are the most common methods utilized to eliminate plant viruses[43]. Plants maintained at 35-45°C normally can survive, while viruses decrease the multiplication rate [2]. In the event that a valuable clone is not available as certified material, it is possible to produce virus-free plants from infected vines, using heat-treatment and meristem culture [1]. 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 [14]. *In vitro* multiplication of grapevine depends on the culture media, the growth regulators [8, 7] and also on the genotype and environmental conditions [6]. Thus, the objective of this experiment was to study for the regeneration potential of *in vitro* cultivated meristems, rooting and potting capacity in five major Iranian grapevine cultivars to obtained free-viruses stocks grapevines. From the economic point of view, after producing healthy plants, it needs a method that can potentially produce large number of healthy vines in a short period.

MATERIALS AND METHODS

Plant materials and heat treatments

Apical shoot-tips were obtained from rooted cuttings of grape stocks in grapevines collection of Kahriz Horticultural Research Station, Urmia, Iran. Cuttings obtained from 15 years-old vines belonging to the "Paykani", "Sultana", "Asgari", "Fakhri" and "Shast Arus" grape cultivars were forced in greenhouse and 4 buds elongated shoots were used as explants sources. Surface sterilization of cuttings was conducted in 2.5% of active chlorine for 15 minutes and rinse with distilled water, then, were rooted in perlite + peat (1:1 in vol.) media.

Establishment of in vitro culture

The shoot segments with apical buds were surface-disinfected for 15 min by continuous agitation in 5% commercial bleach and the buds were aseptically dissected further more to remove all extraneous leaves except for the small appendages directly enclosing the shoot apical meristem.

After surface sterilization, shoot-tips were rinsed constantly three times in sterile distilled water for 5, 10, 15 min. Outer leaves of the apical buds removed in a laminar air flow cabinet, and shoot apices containing 2-3 leaf primordia were then excised. Meristems (0.1-0.2 mm) were isolated under a binocular microscope[39] and inoculated on half-strength MS medium [32]. The medium was supplemented with 0.5 mg L^{-1} of BAP, 0.01 mg L^{-1} of NAA, 30 g L^{-1} of sucrose, and 7.0 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. For shoot multiplication, MS medium containing hormones BAP 0.5mg L^{-1} was used. Rooting of seedling was performed with the medium MS+ BAP 0.1 mg L^{-1} + IBA 1mg L^{-1} + sucrose 20gr L^{-1} (25±3°C, 5000 Lux.). Sub-culturing was performed every 4 weeks to establish a massive mother stock culture before initiating the experiments. Callus weight, root weight *In vitro* derived shoots, excised and pretreated with 1mgL^{-1} IBA, were directly potted in potting mix (2/3 Peat + 1/3 Perlite). Then they were subjected to hardening by covering the plants with polythene bags and glass beaker and maintaining humidity by providing mist spray of water over the plant by removing the cover while keeping in culture room continuously for 15 days in all treatments. In all treatment, the plantlets were kept in continuous light (approx. 800 lux) in culture room at a temperature of 26±2°C. The cover was gradually removed after 6 days initially for 3 hours followed by 6 hours and 12 hours in next 6 days. Subsequently, the period of keeping the plantlets at room temperature was gradually increased and/or increasing holes in the polythene bags with time, after 15 days they were brought outside air-conditioned room.

RNA extraction

Total RNA was extracted from leaves and petioles of 2 shoots of rooted cuttings and 10 grapevine plantlets from meristem culture 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. 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[22].

Detection of GLRaV-3, GLRaV-1, and GFLV by RT-PCR

Total RNA extracted from grapevine tissues developed rooted cuttings and 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 MgSO4, and 1µM 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 5.0µL 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 (10µL 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). A DNA molecular weight marker, a 100 bp DNA ladder, was used to determine the size of the amplified fragments.

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

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

Data 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 SAS software as described by SAS Institute Inc. (2003). Simple linear regression was used to estimate the amount of shoot doubling time.

RESULTS

Meristem culture effects on healthiness of plantlets in grapevine cultivars

At least 79% of the explants were adequately established *in vitro.* **To reducing of viruses infection, response of the cultivars to meristem culture were different.** The highest healthiness (95.5%) observed in Asgari and the lowest (92.5%) in sultana cultivar (fig. 1).

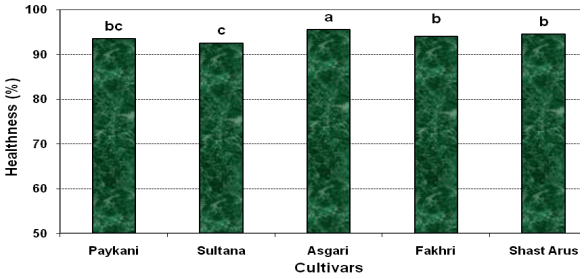

Fig 1.Health plantlet ratio after meristem culture in different cultivars

Explants Establishment and Shoot Proliferation

The data presented in figure 2 reveals significant variation for percent explants establishment of cultivars in MS basal media fortified with same combination of materials. In terms of genotypic response, genotype Sultana was proved remarkably superior to others for the most of cultures phases. Maximum callus induction frequency (fig 4- A) was exhibited by cultivar Paykani (0.4 gr) followed by Fakhri (0.37gr) and others cultivars were in lower ratio (Table 2). The highest explants establishment (86.66%) and least days (30) for explants establishment were observed in MS medium supplemented with 1.0 mgL^{-1} BAP in Sultana compared with Asgari cv.(79.50 percent explants establishment in 30 days) (Fig. 2) (fig 4-C).

Table 2. Influence of Ms Media on some characteristics of meristemic explants regeneration of *in vitro* grown *V. vinifera* cultivars.

Cultivars	Characteristics							
	Callus weight (g)	**Root Fresh weight (g)**	**Root length (mm)**	**Number of Roots**	**Shoot Fresh weight (g)**	**Number of Shoots**	**Number of Leaf**	**Fresh weight Plantlet (g)**
Paykani	0.4a±0.021	0.18c±0.01	3.17ab±0. 12	3.4ab±0.14	1.14a±0.01	3.0 b±0.08	17.4b±0.2	1.33d±0.01
Sultanina	0.19c±0.012	0.26b±0.02	3.22a±0.14	3.8a±0.21	1.11a±0.01	3.75a±0.09	20.8a±0.3	1.37b±0.009
Asgari	0.01d±0.006	0.34a±0.01	2.88b±0.21	3.3b±0.13	1.03c±0.01	3.2b±0.1	17.7b±0.15	1.38ab±0.01
Fakhri	0.37b±0.02	0.29ab±0.03	2.83b±0.12	2.8c±0.12	10.5b±0.02	3.7a±0.04	16.8b±0.24	1.35c±0.008
Shast Arus	0.17c±0.01	0.28ab±0.02	3.17ab±0.14	3.4ab±0.11	1.10ab±0.012	3.7a±0.09	18.2ab±0.32	1.39a±0.009
LSD 5%	0.05	0.09	0.41	0.42	0.04	0.4	2.1	0.032

Values within column followed by different letters are significantly different at 5% probability level.

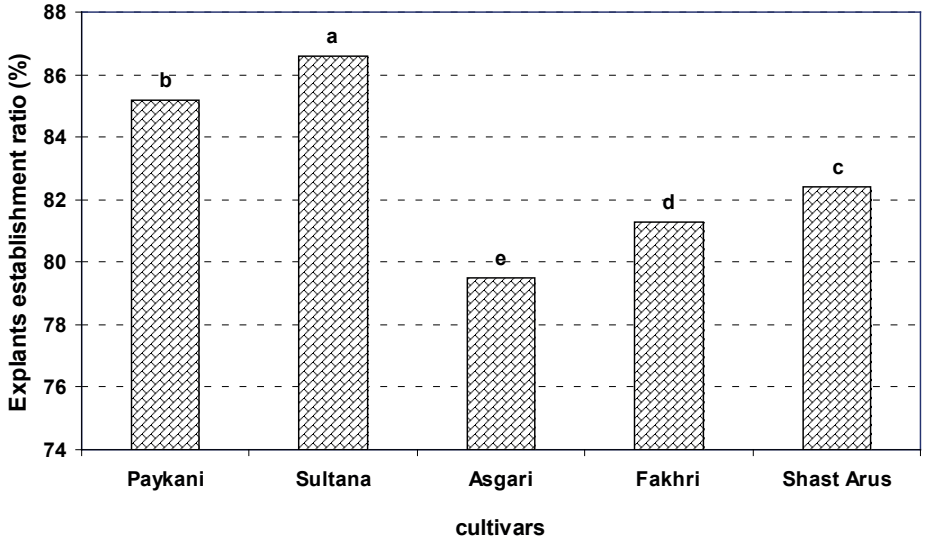

Fig 2. Explants establishment ratio of meristem explants at MS media in different cultivars

Similar results have been reported by Alizadeh (2007) and Mezzetti *et al.* (2002). Shoots in higher numbers also achieved in cultivar Sultana less (3.75) with the medium fortified with combination of an auxin and a cytokinin and others cultivars were in lower ratio, however, higher shoot proliferating ability was shown by cultivar Sultana (86.6%) followed by Paykani (85.2%). Cultivar Paykani (1.14 g) followed by Sultana (1.11g) and Shast Arus less (1.1g) produced shoots of higher weight numerically (Tale 2).

Shoot doubling time is a convenient parameter for use in optimizing proliferation rates in shoot cultures; its use may also facilitate investigations into the mechanisms of processes underlying shoot proliferation *in vitro* (fig. 4-B). Maximum Shoot doubling time was exhibited by cultivar Sultana (4.8) and Fakhri cultivar (4) was in lower ratio (Fig. 3) (fig 4- E).

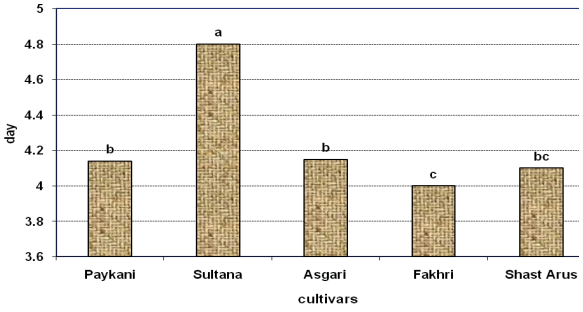

Fig 3. Shoot doubling time of meristem explants at MS media in different cultivars

In vitro rooting

A perusal of data (Table 2) shows significant variation for number, length and fresh weight *in vitro* rooting of five cultivars grape. In same media, an average of 3.8 number rooting was obtained Sultana cv. being highest (3.2 mm) in length followed by Asgari cv. (3.3 numbers) rooting (fig4-D).

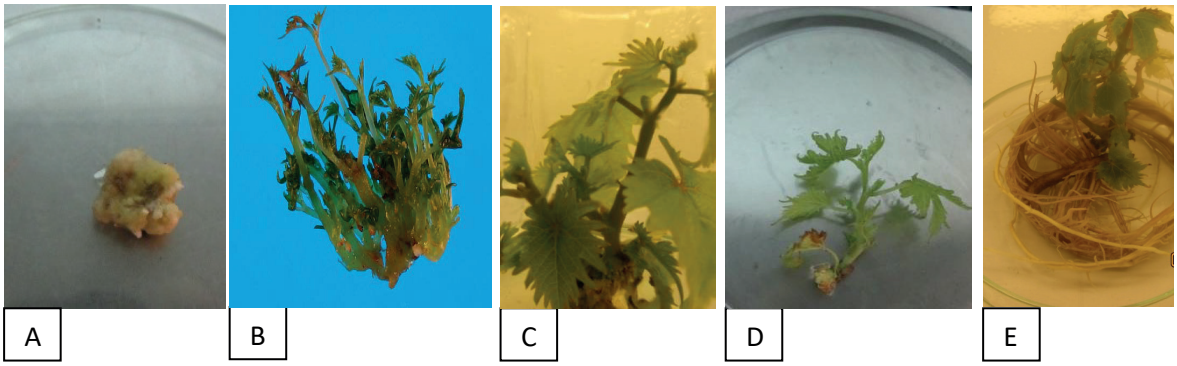

Fig 4. Stages of *in vitro* meristem culture and regeneration organs in MS Medium. A: callus formation, B: proliferation, C: shoot formation, D: root formation, E: complate plantlet

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* meristem culture (Figure 5).

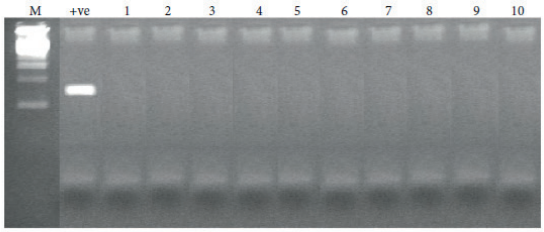

Fig 5. Agarose gel (1.5%) electrophoresis of the coat protein gene of grapevine virus GLRaV-3 amplified by RT-PCR using the primer C2647; H2042. Total RNA extracted from *V. vinifera* cv. "Asgari" grapevine grown *in vitro*. Lines: 1-10, *V. vinifera* grown *in vitro*; M, Molecular weight 1 Kb marker; +ve, positive control of GLRaV-3.

DISCUSSION

Apical meristems contain less endophytic contamination and they are most vigorous during initial rapid growth[1]. Support for this hypothesis comes from preliminary experiments with some slow growing, potted grapes, where *in vitro* proliferation of shoot tip cultures was difficult[8]. The method of elimination of viral diseases, which consists of a combination of thermotherapy and subsequent sampling of apical segments (greater than meristems) has been used since 1960s [14]. Both recent and older studies [13,15,17]dealt with thermotherapy of *in vitro* cultivated grape-vine plants when eliminating the GFLV. However, the thermotherapy *in vivo* (i.e. heat treatment of plants directly in the substrate combined with a subsequent rooting of sampled segments under sterile conditions) has also a number of advantages. Our methodology is based on the reports of other authors about using thermotherapy and apical meristems as explants for *in vitro* grapevine culture establishment [14, 23, 14] and differs from the protocol described by Lee & Wetzstein[21], where axillary buds were used as initial explants. The shoots developed within 4 weeks and the average number of shoots per apex was 3.0-3.75 depends in cultivar. The shoots produced on MS culture media had a normal morphology compared to those from other variants. In a series of previous studies BA has been used for micropropagation in Muscadinia and Euvitis species, hybrids and cultivars [15, 23]. In the present study, plantlets of Sultana and other cultivars of 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 [18, 19].

The cultivars in this study differed in their multiplication and development potential. The effect of the genotype on the various aspects of the performance of tissue-cultured material was also reported in other studies on *Vinifera* cultivars [8, 36]. Our experiments confirmed that multiplication depends not only on the concentration of cytokinins in culture medium, but also on the response of individual genotype. The observed differences in multiplication among grapevine cultivars in this study under the influence of an exogenous BAP could result from the genetic control of different auxin and cytokinin metabolisms of plant tissue. The studies of Barlass and Skene[5] on the *in vitro* culture of a range of *Vitis* cultivars, breeding lines and species showed clear differences in genotypic multiplication rates and cultural behavior which suggest a link between multiplication rate and genetic composition related to geographical origin.

In the present experiment the BAP treatment produced rooted shoots with significantly more roots per shoot compared between cultivars. In vitro rooting studies demonstrated that medium with 1mgL-1 IAA significantly increased percentage of rooted shoots, but root lengths were smaller compared with in vivo conditions. Root formation was diminished or inhibited with the presences of cytokinins. This agrees with previous findings obtained on grapes [23, 37, 35. 9]. The addition of cytokinin to the medium led to increased leaf numbers, number of new shoots, and shoot length in grapes (fig 2)[35, 9]. The stimulatory effects of IAA on adventitious rooting of in vitro produced grape shoots have been previously described [6]. All cultivars of *V. vinifera* in this study, up to 90% rooting of plantlets were obtained on MS medium supplemented with 1mgL^{-1} IAA. Also, in our experiment the best shoot proliferation medium (MS with 1 mgL^{-1} BAP) re-

sulted in shoots that were successfully rooted in vivo, and subsequently decreased plantlet acclimatization period. Similar to our results an optimum rate of shoot proliferation was also reported in different grape cultivars by other researchers [40, 31]. However, following our studies, tissue culture for free-viruses stocks of grapevine and useful propagation protocol, characterized by higher regeneration efficiency was simply achieved by in vitro manipulations. It is possible that isolating individual buds *in vitro* with no tissue attached increases the acquisition of healthy plants; nevertheless, when small explants is used, the chances of recovering a complete plant are reduced [41, 20]. 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[1] demonstrated the value of meristem culture 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*. In our case, we obtained a high rate of virus-free plants using relatively large explants, ensuring the procedure's success by meristem culture.

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