International Journal of Agriculture and Environmental Research

ISSN: 2455-6939

Volume:02, Issue:06

In vitro REGENERATION OF VIRUS-FREE GRAPEVINE (*Vitis vinifera* L.)

Hossein Nouri[1], Valiollah Rasoli[2*], Vahid Abdousi[3]

[1]MSc. Student of Horticultural sciences, Faculty of Agriculture, Islamic Azad University, Science and Research Brach of Tehran, Tehran, Iran. Email: hnoori19@yahoo.com

[2]Assistance Professor (Ph.D.), Horticulture Crops Research Department, Qazvin Agricultural and Natural Resources Research and Education Center, AREEO, Qazvin, Iran. Email: Spiiqv@gmail.com

[3]Horticultural sciences Department, Faculty of Agriculture, Islamic Azad University, Science and Research Brach of Tehran, Tehran, IRAN

ABSTRACT

Grapevines (*Vitis vinifera* L.) are very susceptible to virus diseases. In order to determine the usefulness of thermotherapy (50°C and a gradual increase at 10°C and humidity 80% for 30 minutes) and *in vitro* meristem culture to obtain virus-free grapevine plants were used in five grape cultivars (*Vitis vinifera*)"Paykani" , "Sultanina", "Kondori", "Fakhri" and "Shast Arus". Grapevine leaf extract samples were initially tested by indicator plants to confirm their virus status. Afterward, the apical meristems from the plants surviving the thermotherapy treatment 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 and all the plants that survived thermotherapy were assessed for their virus status using indicator plants. Results showed BAP at 0.5 mg L^{-1} gave the highest *in vitro* multiplication rate, with 3.75 shoots per explant, whereas elongation was greatest in the presence of 0.5 mgL^{-1} of BAP. The mean percentage of free disease was in Fakhri 94%, Peykani 93.5%, Sultana 92.5%, Kondori 95.5% and Shast Arus 94.5%. The results showed that the used method had high reliable in the propagation and production of disease-free plant.

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

1. 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

ISSN: 2455-6939

Volume:02, Issue:06

development and profitable production of viticulture (Martelli, 2006; Weiland et al., 2004). Grapevines diseases are caused by intracellular pathogens of different natures, among which plant viruses are the most important (Cretazzo et al., 2010; Skiada et al., 2013).Plants infected with grape viral diseases show less vigorous growth and delayed bud opening, and may decline and die within a few years. 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) (Martelli and Boudon-Padieu, 2006). The prevalence of the virus is very high in both simple and mixed infections with other more dangerous viruses (Cretazzo et al., 2010; Kominek et al., 2005). In recent past, it has been observed that non-availability of adequate number of true to type; disease free planting material has been the major constraint for establishment of ideal vineyards in Iran. One basic drawback of conventional methods, however, is that they don't allow a rapid buildup of grape material that is in limited supply (Walter and Martelli, 1998). More and more often today as a result of clonal selection programs, as unique virus-free clones are identified by grape virologist, or as new varieties are produced by grape breeders, there is a need for the rapid buildup of unique techniques so that vines may be available in sufficient quantities for commercial production (Raymond et al., 1984). Thermotherapy and meristem culture are the most common methods utilized to eliminate plant viruses. Plants maintained at 35-45°C normally can survive, while viruses decrease the multiplication rate (Mannini et al., 1998). *In vitro* morphogenesis, however, appears to be dependent on the interaction between genotype, explant source and culture medium, and thus, it is necessary to develop specific regeneration protocols for each *Vitis* cultivar (Mannini et al., 2003).Tissue culture results in rapid clonal multiplication and uniformity in obtained clones, vigorous growth, normal yield and healthy plants (Alizadeh, 2007). 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 (Abu Shirbi, 2001). 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 Goheen and Luhn (1973), meristem culture and thermotherapy are the most often employed methods for obtaining virus-free grapevine plants (Infante and Fiore, 2009). *In vitro* multiplication of grapevine depends on the culture media, the growth regulators (Chee and Pool, 1983; Butiuc-keul et al., 2009) and also on the genotype and environmental conditions (Harris and Stevenson, 1982). *In vitro* culture of grapevine was also used for production of virus free plantlets (Muhammad et al., 2008), and somatic embryogenesis as well (Barreto and Nookaraju, 2007).Thus, the objective of this experiment was to study for the first time the regeneration potential of *in vitro* cultivated meristems and *in vitro* rooting and potting capacity in five major Iranian grapevine cultivars in order 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

ISSN: 2455-6939

Volume:02, Issue:06

vines in a short period. To achieve this, in this study we described a simple and rapid method by which large scale of adventitious buds were proliferated from singe fragmented shoot apex of some Iranian grapevine cultivars.

2. MATERIALS AND METHODS

2.1. Plant materials

Apical shoot-tips were obtained from meristem culture of grape stocks in grapevines collection of Takestan Viticulture Research Station, Qazvin, Iran. Cuttings obtained from 8 years-old vines belonging to the "Paykani", "Sultanina", "Kondori", "Fakhri" and "Shast Arus" grape cultivars were forced in greenhouse and 3 buds elongated shoots were used as explant sources. Heat treatment was done with a maximum temperature of 50°C and a gradual increase at 10°C and humidity 80% for 30 minutes in the incubator. Surface sterilization of explants was conducted in 2.5% of active chlorine for 15 minutes and 70% ethanol for 15 seconds and rinse with distilled water. After rooting cuttings and growth shoots in perlite medium, also, the shoot segments with apical and axillary 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.

2.2. Establishment of in vitro culture

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 (Shatnawi et al. 1999) and inoculated on half-strength MS medium (Murashige and Skoog 1962). 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

ISSN: 2455-6939

Volume:02, Issue:06

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.

2.3. Detection of virus infection in plantlets

In the spring of the second year the over wintered plantlet were growth and leaf extract occasionally checked also by mechanical transmission onto stem of herbaceous indicator plants: *Chenopodium quinoa*, *C. amaranthicolor*, *Cucumis sativus* cv. "Delicates", *Gomphrena globosa*, *Nicotiana clevelandii*, *N. tabacum* cv. "Samsun", *N. glutinosa*, *Phaseolus vulgaris* cv. "Beautiful" and *Cucumis sativus*. At the end, the marked grapevine plants, giving negative results on all indicators were considered virus-free (Lázár *et al*., 2002).

2.4. Data analysis

The data were analyzed according to completely randomized block design (CRD) as described by SAS Institute Inc. (2003). Mean comparison was done by Duncan's multiple range tests at 0.05% probability level. Simple linear regression was used to estimate the amount of shoot doubling time.

3. RESULTS

3.1. Heat treatment and meristem culture effects on healthiness of plantlets

To reducing of viruses infection, response of the cultivars to thermotherapy and meristem culture were different, results showed in all cultivars thermotherapy had lower effect to viruses infection reducing in plantlets (fig. 1). The highest healthiness (95.5%) observed in Kondori, when meristem culture used compared with same cultivar (65.25%) treated by thermotherapy. At least 79% of the explants were adequately established *in vitro*. Losses due to contamination were low, since the mother plants came from controlled growth conditions and the fungal and bacterial load was less than for plants from the field.

3.2. Explants Establishment and Shoot Proliferation

The data presented in Table 1 reveals significant variation for percent explants establishment of cultivars in MS basal media fortified with same combination of materials. In terms of genotypic

ISSN: 2455-6939

Volume:02, Issue:06

response, genotype Sultanina was proved remarkably superior to others for the most of cultures phases. Maximum callus induction frequency was exhibited by cultivar Paykani (0.4 gr) followed by Fakhri (0.37gr) and others cultivars were in lower ratio (fig. 2). Similar results have been reported by Alizadeh (2007) and Mezzetti et al. (2002).Shoots in higher numbers also achieved in cultivar Sultanina less (3.75) with the medium fortified with combination of an auxin and a cytokinin, however, higher shoot proliferating ability was shown by cultivar Sultanina (86.6%) followed by Paykani (85.2%). Cultivar Paykani (1.14 gr) followed by Sultaniana (1.11gr) and Shast Arus less (1.1gr) produced shoots of higher weight numerically (Tale 1).

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 (Chee and Pool, 1983 and Raymond et al., 1984). 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 et al. (1980) 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.

Table 1. Influence of Ms Media on some characteristics of meristemic explants regeneration of *in vitro* grown *V. vinifera* cultivars.

Cultivars	Characteristics									
	Explants establishment ratio (%)	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)	Shoot doubling time (number/30day)
Paykani	85.2a±0.078	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	4.14b±0.01
Sultanina	86.6a±0.021	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	4.8a±0.01
Kondori	79.5c±0.041	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	4.15b±0.008
Fakhri	81.3b±0.032	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	4c±0.009
Shast Arus	82.4b±0.016	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	4.1b±0.007
LSD 5%	1.25	0.05	0.09	0.41	0.42	0.04	0.4	2.1	0.032	0.03

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

ISSN: 2455-6939

Volume:02, Issue:06

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 Sultanina compared with Kondori cv.(79.50 percent explant establishment in 30 days) (Table 1). 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. Maximum Shoot doubling time (number/30day) was exhibited by cultivar Sultanina (4.8) followed by other cultivars (0.37gr) and Fakhri cultivar (4) was in lower ratio (Table 1).

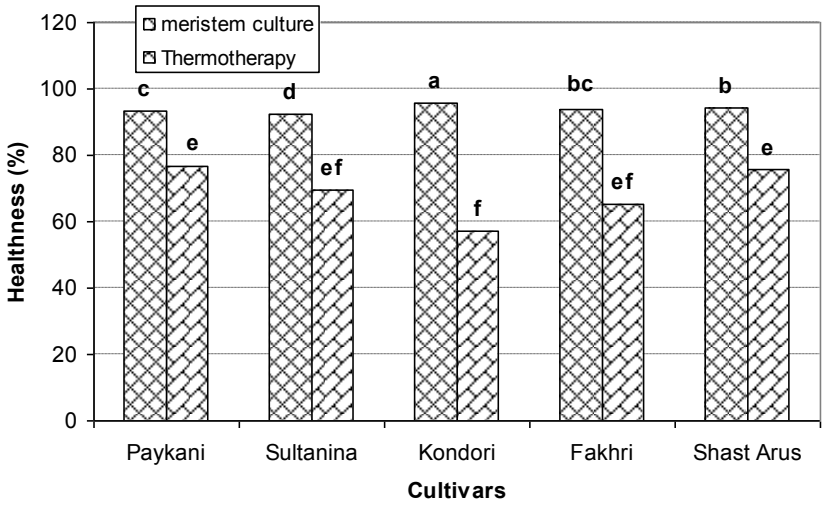
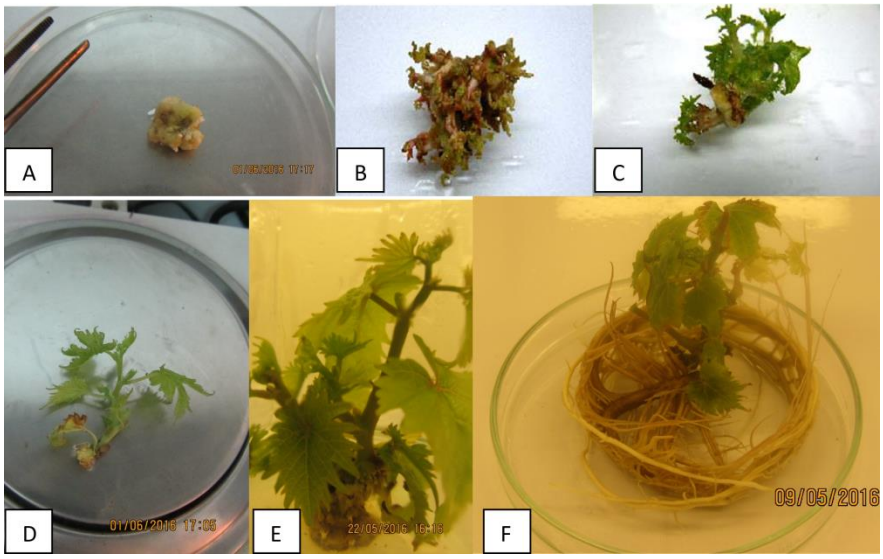


Fig. 1: Health plantlet ratio after thermotherapy and meristem culture in different cultivar



ISSN: 2455-6939

Volume:02, Issue:06

Fig 2. Stages of *in vitro* culture and regeneration organs in MS Medium. A: callus formation, B: proliferation, C: shoot formation, D: root formation, E: shoots growth, F: complate plantlet

3.3. In vitro rooting

A perusal of data (Table 1) 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 Sultanina cv. being highest (3.2 mm) in length followed by Kondori cv. (3.3 numbers) rooting. 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 (fig 2)(Pathirana and McKenzie 2005; Couselo et al. 2006).

4. DISCUSSION

Thermotherapy is one pathway for producing free-virus and endophytic contamination plant in woody plants. Also, Apical meristems contain less endophytic contamination and they are most vigorous during initial rapid growth (Abu Shirbi, 2001). Support for this hypothesis comes from preliminary experiments with some slow growing, potted grapes, where *in vitro* proliferation of shoot tip cultures was difficult (Chee and Pool, 1983). 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 (Gua, 2010). Both recent and older studies (Goheen and Luhn, 1973; Harris and Stevenson, 1982; Křižan et al., 2008) 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 (Gua, 2010; Panattoni et al., 2007; Panattoni and Triolo, 2010) and differs from the protocol described by Lee & Wetzstein (1990), 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 (Harris and Stevenson, 1982; Mannini et al., 2003).

ISSN: 2455-6939

Volume:02, Issue:06

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. The stimulatory effects of IAA on adventitious rooting of *in vitro* produced grape shoots have been previously described (Chee et al., 1984; Barreto and Nookaraju, 2007). 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) resulted 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 (Skiada et al., 2013).; Muhammad et al., 2003). However, following our studies, the combined method of thermotherapy and tissue culture for free-viruses stocks of grapevine and usefull 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 explant is used, the chances of recovering a complete plant are reduced. In our case, we obtained a high rate of virus-free plants using relatively large explants, ensuring the procedure's success.

REFERENCES

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

2. Alizadeh, M. (2007). Micropropagation and *in vitro* screening of some grape (*Vitis* spp.) rootstock genotypes for salt tolerance. Ph.D. thesis submitted to the P.G. School, Indian Agricultural Research Institute. New Delhi, India

3. Barlass, M., & Skene, G.M. (1980). Studies on the fragmented shoot apex of grapevine. II. Factors affecting growth and differentiation *in vitro*. J.Exp. Bot., 31: 489–95

4. Barreto, M. S., & Nookaraju, A. (2007) Effect of auxin types on *in vitro* rooting and acclimatization of grapevine as influenced by substrates. Ind. J. Hort., 64: 5-11.

5. Butiuc-keul, A. L., Coste, A., Halmagyi, A., Deliu, M., Farago, M., & Iliescu, R. (2009). *In vitro* micropropagation of several grapevine cultivars from Romania. Acta Hort., 812 (1), 133-138.

ISSN: 2455-6939

Volume:02, Issue:06

6. Chee, R., & Pool, R. M. (1983). *In vitro* vegetative propagation of *Vitis*: Application of previously defined culture conditions to a selection of genotypes. Vitis, 22, 363-374.

7. 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.

8. Cretazzo, E., Tomas, M., Padilla, C., Rossello, J., Medrano, H., Padilla, V., & Cifre, J. (2010). Incidence of virus infection in old vineyards of local grapevine varieties from Majorca: implications for clonal selection strategies. Span J Agric Res, 8(2):409-418.

9. Goheen, A.C., & Luhn, C.F. (1973) Heat inactivation of viruses in grapes. Rivista di Patologia Vegetale, 9, 287–289.

10. Gua, I.C. (2010). Alternative method for obtaining virus-free grapevine propagating material. Univ. of Agronomic Science and Veterinary Medicine Bucharest, PhD Thesis, 208 p.

11. Harris, R. E., & Stevenson, H. (1982). In vitro propagation of *Vitis*. Vitis , 21, 22-32

12. Infante, R., &Fiore, N. (2009). Combined effect of thermotherapy and in vitro shoot culture on the Grapevine leafroll associated virus 2 Red Globe strain affecting 'Red Globe' vines. 2009. Journal of Food, Agriculture & Environment , 7 (3&4): 2 7 4 - 2 7 7.

13. Křižan, B., Ondrušiková, E., Holleinová, V., Moravcová, K., & Bláhová, L. (2009). Elimination of grapevine fanleaf virus in grapevine by *in vivo* and *in vitro* thermotherapy. Hort. S CI . (PRAG ue), 6(3): 105–108 .

14. Kominek, P. (2008). Distribution of grapevine viruses in vineyards of the Czech Republic. J Plant Pathol, 90(2):357-358

15. Komínek, P., Glasa, M., & Bryxiová, M. (2005). Analysis of the molecular variability of grapevine leafroll-associated virus 1 reveals the presence of two distinct virus groups and their mixed occurrence in grapevines. Virus Genes, 31: 247-255

16. Lázár, J., Mikulás, J., Farkas, G., & Kölber, M. (2002) Certification program for production of virus-free propagating material of grapevine and its results in Hungary. International Journal of Horticultural Science, 8: 39-43

ISSN: 2455-6939

Volume:02, Issue:06

17. Lee, N., & Wetzstein, H.Y. (1990). *In vitro* propagation of muscadine grape by axillary shoot proliferation. J. Amer. Soc. Hort. Sci., 115: 324-9.

18. Mannini, F., Gerbi, V., & Credi, R. (1998). Heat-treated v. virus-infected grapevine clones: Agronomical and enological modifications. Acta Horticulturae, 473 :155-164.

19. Mannini, F., Rolle, L., & Guidoni, S. (2003). Vineyard management to optimize grape quality in virus-free clones of *Vitis vinifera* L. Acta Horticulturae, 603, 121–126.

20. Martelli, G. p., & Boudon-Padieu, E. (2006a). Directory of infectious diseases of grapevines. In: Options mediteraneenes. Serie B: Studies and Research, 55, 280 p.

21. Martelli, G. P., & Boudon-Padieu, E. (2006b). Directory of infectious diseases of grapevines and viruses and virus-like diseases of the grapevine: Bibliographic Report 1998-2004. 1st edition, CIHEAM. Bari, 279 pp

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

23. Muhammad, J., Jaskani, H. A., Sultana, R., Khan, M.M., Qasim, M., & Khan, I. A. (2008). Effect of growth hormones on micropropagation of *Vitis vinifera* L cv. Perlette. Pak. J. Bot., 40: 105-109

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

25. Panattoni, A., D'Anna, F., Cristani, C., & Triolo, E. (2007). Grapevine virus eradication in *Vitis vinifera* explants by antiviral drugs and thermotherapy. J Virol Methods, 146:129-135.

26. Panattoni, A., & Triolo, E. (2010). Susceptibility of grapevine viruses to thermotherapy on *in vitro* collection of Kober 5BB. Scientia Horticulturae, 125:63-67

27. 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.

28. Raymond, C., Pool, R.M., & Bucher, D. (1984). A method for large scale *in vitro* propagation of *Vitis*. New York's Food and Life Sci. Bul., 119: 1–9

ISSN: 2455-6939

Volume:02, Issue:06

29. Salunkhe, C. K., Rao, P. S., & Mhatre, M. (2004). Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera* L. Pla Cell Rep, 17: 56-67.

30. SAS INSTITUTE INC. (2003). The SAS system for windows, release 8.2 SAS Institute Inc., Cary, NC, USA.

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

32. Valero, M., Ibañez, A., & Morte, A. (2003). Effects of high vineyard temperatures on the grapevine leafroll associated virus elimination from *Vitis vinifera* L. cv. Napoleon tissue cultures. Scientia Horticulturae, 97 :289-296.

33. Walter, B., & Martelli, G.P. (1998). Considerations on grapevine selection and certification. Vitis, 37, 87–90.

34. Weiland, C.M., Cantos, M., Troncosso, A., & Perez-Camacho, F. (2004). Regeneration of virus-free plants by in vitro chemotherapy of GFLV (Grapevine Fanleaf Virus) infected explants of *Vitis vinifera* L. cv. Zalema. Acta Hort, 652:463-466.

