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Original article

Effects of shoot tip size on *in vitro* regeneration and virus elimination of grapevine cv. Superior Seedless

"Superior Seedless" üzüm çeşidinde sürgün ucu büyüklüğünün *in vitro* rejenerasyon ve virüs eliminasyonu üzerine etkileri

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ABSTRACT

In this study, we investigate the relation between the explant size and the success of virus elimination in 'Superior Seedless' grape variety. Plants were tested by prior to thermotherapy and *in vitro* culture by using Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay (DAS-ELISA) and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and verified as infected by different viruses (*Grapevine leafroll associated virus -1+3, Grapevine leafroll associated virus -4 Strains, Grapevine rupestris stem pitting associated virus*). Different size of shoot tip explants (0.1-0.5-1-5-10 mm) were excised and cultured on C_2D medium supplemented with 1 mgl^{-1} BA. All of the explants bigger than 0.1 mm 100% regenerated while 70% of the 0.1 mm explants showed complete regeneration and turned into a whole plant. Plants regenerated from 10 mm shoot tip explants have all three viruses (GLRaV-1+3, GLRaV-4 strains and GRSPaV) as founded in mother plant. Plants regenerated from 5 mm explants have two of them (GLRaV-1+3, GLRaV-4 strains) and plants regenerated from 1 mm explants have one (GLRaV-1+3). Plants regenerated from 0.1 mm explants were found completely virus-free.

INTRODUCTION

Sanitary status of the vineyard is crucial and it is important to use virus-free plant material for both satisfactory yield and quality and for conservation of local varieties, for correct amphelographic characterization and to reveal genetic potential and hence successive breeding. Virus symptoms may be different depending on the virus type and the plant species. Some may cause shape and colour disorders in shoots and leaves while the others may cause growth retention, yield loss and death. Methods (meristem and shoot tip culture, somatic embryogenesis, micro grafting, thermotherapy, cryotherapy, chemotherapy, electrotherapy etc.) for obtaining virus-free plant material are primarily based on tissue culture techniques and could be used single or combined. The most common methods are meristem or shoot tip culture and thermotherapy. Shoot tip culture was first used by Morel and Martin (1952) to clean virus infected dahlia plant (*Dahlia* sp.). Since then it is routinely being used. Meristematic zone is usually free from viruses or contain significantly lower virus concentration (Gribaudo et al. 2006). Smaller size than 0.1 mm explants referred as

meristem culture. Practically 0.1-0.2 mm size explants are used. Explant size is critically important in virus elimination. The smaller the explant the more success the elimination of pathogens. On the other hand, smaller explants have less ability to regenerate (Infante and Fione 2009, Maliogka et al. 2009, Skiada et al. 2009). Thermotherapy refers *in vivo or in vitro* heat application for several weeks in 38 °C may also be effective to keep viral proteins in infected tissue and prevent the virus to reach shoot tips by slowing down the movement (Mink et al. 1998). In our laboratory, production of virus-free base material by combining these two methods is routinely being done. In this study, we aimed to investigate the relation between the shoot tip size and the effectiveness of virus elimination in 'Superior Seedless' grape variety and to determine the optimum explant size to have virus-free and highly regenerative explants.

MATERIALS AND METHODS

The study was conducted at Manisa Viticulture Research Institution between the years 2018 and 2019. 'Superior Seedless' (*Vitis vinifera* L.) grape variety was used in this study. Mother plants were tested prior to thermotherapy and *in vitro* culture for *Grapevine rupestris stem pitting associated virus* (GRSPaV), *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll associated virus-1+3* (GLRaV-1+3), *Grapevine leaf roll associated virus-2* (GLRaV-2), *Grapevine leaf roll associated virus-4 strains* (GLRaV-4 strains), *Grapevine virus* A (GVA), *Grapevine fleck virus* (GfkV) by ELISA and/or RT-PCR methods (Table 1).

ELISA tests were conducted according to Double Antibody Sandwich Method as reported by Clark and Adams (1977). PCR was conducted with Roche LightCycler® Nano Real-time PCR system according to manufacturer's guide. For GLRaV-1 / GLRaV-3, Roche FastStart Essential DNA Green Master Mix consisting SYBR Green I and for GRSPaV, RealTime ready RNA Virus Master Probe based kits were used. PCR protocols were indicated on Table 2.

Infected plants were transferred to 10 l pots and placed in thermotherapy cabin for 12 weeks under 16 h photoperiod and 38/34 °C day/night temperatures. End of the January 2019, 1.5-2 cm apical and axillary shoot tips were excised from the plants in thermotherapy cabin. Then a surface sterilization protocol (wash 15 min under tap water with a few drops of detergent, 10 sec 70% ethanol, wash under tap water, sterilize in 5% sodium hypochlorite with 0.1% Tween for 10 min and rinse 3 times with sterile distilled water) was applied. Four different size of explants (0.1-1-5-10 mm) 22 for the 0.1 mm and 11 for each of the others were excised and cultured on petri dishes contain 20 ml C_2D medium (Chée et al. 1984), supplemented with 3% sucrose, 0.7% Difco-Bacto agar and 1 mg l^{-1} BA (N6-Benzyladanine). pH was adjusted to 5.8 prior to autoclaving at 121 °C, 105 Pa for 15 min. Petri dishes were placed in a culture room under 3000 lux, 16 h photoperiod and 25 °C temperature. 8 weeks of culture was needed for the 0.1 mm explants to regenerate while the bigger ones transferred to fresh medium after one month. Same medium formulation was used for sub-

Table 1. Virus analysis results of the mother plant prior to thermotheraphy and in vitro culture.

Sample	ArMV / FLV*	GLRaV-1+3**	GLRaV-2	GLRaV-4 rains***	GVA	GfKV	GRSPaV
Mother plant	Negative	Positive	Negative	Positive	Negative	Negative	Positive

Table 2. PCR protocols for GLRaV-1 / GLRaV-3 and GRSPaV

Name of primers	Primers (5' 3') and Probes	Size	Type of analyze	PCR cycles
GLRaV-1_9952 / F GLRaV-1_10420 / R	GAGCGACTTGCGACTT ATCGA GGTAAACGGGTGTTCT TCAATTCT (Unpublished)	469 bp	SYBR Green based RT-PCR	1 X (95°C 10 min) 45 X (95°C 20 sec/56°C 20 sec/72°C 47 sec)
GLRaV-3_ LC1 / F GLRaV-3_ LC2 / R	CGCTAGGGCTGTGGAA GTATT GTTGTCCCGGGTACCA GATAT (Osman and Rowhani, 2006)	546 bp	SYBR Green based RT PCR	1 X (95 °C 10 min) 50 X (95°C 20 sec/58°C 20 sec/72°C 55 sec)
GRSPaV/SO / F GRSPaV/SO// R	GCTTGARCCWAAGGG TGGA CCAGCMGTTCCRCCAC TAAT GCTGAAGG (Önder et al., 2016)	150 bp	One Step UPL based RT-PCR	1 X (50°C 480 sec) Reverse transcription 1 X (56°C 240 sec) Reverse transcription 1 X (95°C 30 sec) 45 X (95°C 10 sec/55°C 30 sec/72°C 10 sec)

cultures. During sub-culturing, leaf samples were taken from each plantlet (39 from 0.1 mm, 66 from 1mm, 66 from 5 mm, 96 from 10 mm) from each group and kept in 4 °C for a few days until virus analysis. According to virus analysis results only virus-free plantlets were taken to rooting step and all others were discarded. 27 virus-free plantlets (from 0.1mm explants) successfully rooted and were transferred to green house. During 2019 dormancy period, they were all tested for mentioned viruses and proved as virus-free.

RESULTS AND DISCUSSION

All of the explants bigger than 0.1 mm 100% regenerated while 70% of the 0.1 mm explants showed complete regeneration and turned into a whole plant, 30% of them still green, viable but dormant. Many researchers studied *in vitro* meristem culture of grapevines obtained similar results (Aazami 2010, Gray and Fisher 1985, Gray and Klein 1987 Salami et al. 2009). Laslo et al. (2010), reported 75% regeneration rate of shoot tip explants for 'Cabarnet Sauvignon' and 60% for 'Riesling Italian' grape varieties following 8 weeks of i*n vitro* culture. In a study on 9 different varieties belong *to Vitis rotundifolia*, best media for meristem regenerations varied depending on the varieties and different regeneration rates were obtained in the same medium (Gray and Benton 1991). Previous study on virus elimination efficiency and meristem regenerations of grapevine cv. 'Bornova Misketi' clones revealed that the regeneration rates of the clones varied from 47 to 100% (Ulas et al. 2017). As reported in previous studies, regeneration capacity depends on the plant variety and species (Peros et al. 1998). Genetic variability of *Vitis vinifera* cultivars and clones affect the regeneration capacity of the meristems and elimination efficiency of the procedure (Gray and Benton 1991, Laslo et al. 2010, Peros et al. 1998, Roubelakis-Angelakis and Zivanovitc 1991, Torregrosa and Bouquet 1996). Smerea et al. (2010) reported that the healthy status of grapevine also affected the meristem regeneration capacity. Turcsan et al. (2020) concluded that the meristem culture was a difficult technique as the regeneration capacity of the shoot tips bigger than 0.5 mm is higher but extra length also increase the virus level.

Mother plant was found positive for GLRaV-1+3, GLRaV-4 strains and GRSPaV. After 12 weeks of thermotherapy and *in vitro* culture virus tests were repeated on plants regenerated from the different size of explants. It has been found a relation between explant size and virus composition. Plants regenerated from 10 mm shoot tip explants have all three viruses (GLRaV-1+3, GLRaV-4 strains and GRSPaV) as founded in mother plant. Plants regenerated from 5 mm explants have two of them (GLRaV-1+3, GLRaV-4 strains) and plants regenerated from 1 mm explants have one (GLRaV-1+3). Plants regenerated from 0.1 mm explants were found completely virus-free (Table 3). It has been reported in some studies that elimination of GLRaV-1 was easier than that of GRSPaV-1 with both meristem-tip and shoot-tip culture (Gribaudo et al. 2006, Mannini 2003, Skiada et al. 2009). Here all explants bigger than 0.1 mm have GLRaV-1+3 may due to concentration of the virus in the mother plant or its translocation pattern as all of them are phloem-restricted viruses (Digiaro et al. 1999). As reported in a previous study where woody plant medium supplied with benzyl amino purine (BAP) (1.5 mg l^{-1}) for shoot proliferation was used, 1 mm meristems were found to be optimum to eliminate GLRaV-1 from infected grapevines cv. 'Thompson Seedless' while 3 mm meristems were revealed to carry virus particles. 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 (Youssef et al. 2009).

Table 3. Virus presence in different size of explants after thermotheraphy and in vitro culture

Explant size	GLRaV-1+3	GLRaV-4 Strains	GRSPaV
0.1 mm	negative	negative	negative
1 mm	positive	negative	negative
5 mm	positive	positive	negative
10 mm	positive	positive	positive

Virus eradication in grapevines cv. 'Plavac mali' by using *in vitro* thermotherapy at 39 °C for 32 days and apical bud culture techniques, 26% of all regenerated plants were free from GLRaV-3. However, in different clones virus eradication success were different. Some of the clones were reported as 50% free from GLRaV-3, but in clone OB214 it was not eradicated. Similarly, GLRaV-1 was present in all regenerated plantlets of clone OB091. Virus composition and different endogenous plant regulators of different clones influences the selective virus elimination in grapevines (Hancevic et al. 2015). In another study, elimination of *Grapevine leafroll associated virus-4 Strain Pr* (GLRaV-Pr, Closteroviridae) were carried out in two grapevine cultivars, 'Mantilaria' and 'Prevezaniko', co-infected with GRSPaV (Flexiviridae). Both viruses were detected by nested RT-PCR assays. Researchers combined *in vitro* thermotherapy with meristem (≤ 0.2 mm) or shoot tip culture (≤ 0.5 cm). They found the survival and the regeneration rate of meristems was very low. On the other hand, high survival rates were observed in the cultured shoot tips accompanied with high elimination rates for both viruses. They also reported that virus elimination depends on the genotype of grapevine (Maliogka et al. 2009). Researchers found that the different source of the buds affects the *in vitro* survival rates and virus

free frequencies of grapevines. Survival rates of the shoots were higher when *in vitro* cultures were established from the middle and basal buds than from the terminal buds. Similarly, GLRaV-3- free frequencies were lower in the third axillary shoot tips than terminal, first and second axillary shoot tips (Valero et al. 2003, Wang et al. 2018).

In this study we found a positive relationship between the size of explant and *in vitro* regeneration capacity and also virus-free frequencies of the grapevine. The smaller explants (≤ 1 mm) have less or no virus, also exhibited less regeneration capacities than the bigger ones. It is known that, type of the virus and the grapevine variety also affects these phenomena. Here it may concluded that the virus concentration and the localization patterns affect the sanitary attempts. Further studies will be elucidatory to define these aspects in terms of grapevines sanitary status.

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ÖZET

Bağcılıkta, hücre içi patojenlerin neden olduğu en önemli hastalık etmeni virüslerdir. Bulaşık bitkiler zayıf gelişim, uyanmanın gecikmesi, verim ve kalitede azalma gibi belirtiler gösterebilir hatta birkaç yıl içerisinde ölebilir. Bitkileri virüslerden arındırmak için bazı yöntemler mevcuttur. Termoterapiyle birlikte uygulanan sürgün ucu kültürü en yaygın yöntemdir. Yöntemin başarısı eksplantın büyüklüğüne bağlıdır yani, eksplant ne kadar küçük olursa o kadar fazla virüsten ari bitki elde edilebilir. Öte yandan küçük eksplantların rejenerasyon kapasiteleri düşüktür. Bu çalışmada, 'Superior Seedless' üzüm çeşidinde eksplant büyüklüğü ile virüs eliminasyonu arasındaki bağlantı incelenmiştir. Bitkiler termoterapi ve *in vitro* kültür öncesinde, Double Antibody Sandwich-Enzyme-Linked Immunosorbent Assay (DAS-ELISA) ve Real-time Reverse Transcription-Polymerase Chain Reaction (Real time RT-PCR) teknikleri ile test edilmiş ve farklı virüsler (Grapevine leafroll associated virus -1, -3, Grapevine leafroll associated virus -4 strains, Grapevine rupestris stem pitting associated virus) ile bulaşık oldukları doğrulanmıştır. Farklı büyüklükte (0.1-0.5-1-5-10 mm) sürgün ucu eksplantları, 1 mgl^{-1} BA içeren C_2D ortamında kültüre alınmıştır. Küçük boyutlu eksplantlarda virüs bulaşıklığı az olmuş ya da hiç saptanmamıştır.

Anahtar kelimeler: *in vitro*, eksplant büyüklüğü, virüs eliminasyonu, rejenerasyon, asma

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