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Elimination of Grapevine Virus A (GVA) by Cryotherapy and Electrotherapy

Sh. Bayati[1], M. Shams-Bakhsh[1*], and A. Moieni[1]

ABSTRACT

The incidence of grapevine virus A (GVA) is reported from almost all of the major grapevine growing regions in Iran. Grapevine is vegetatively propagated by rooting of cuttings or grafting. In such plants, viral diseases are transmitted from stock plants to the progeny. Therefore, the control of grapevine viruses can be achieved primarily through production of healthy stock plants. In the present research, cryotherapy and electrotherapy were employed for elimination of GVA from naturally infected vine (*Vitis vinifera* L. cv Black) and their efficiency was compared. In cryotherapy, 59% of the shoot tips survived and regenerated into whole plants, of which 42% were free of GVA detected by RT-PCR. In the electrotherapy, the effects of electric current value and treatment duration were investigated on plant survival and virus elimination. The best results were obtained by using 30 milliamps (mA) for 15 minutes. With this treatment, survival and virus-free frequencies were about 62% and 40%, respectively. This is the first report of electrotherapy of grapevine shoot tips as a potential tool for GVA elimination. The results showed that cryotherapy was a more efficient and convenient protocol than electrotherapy for elimination of GVA from infected grapevine.

Keywords: Cryotherapy, Electrotherapy, RT-PCR, Shoot tips, *Vitis*

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an economically important crop in Iran that is at the risk of serious diseases caused by viruses and virus-like pathogens, transmitted and perpetuated by vegetative propagation (Martelli and Boudon, 2006).

Grapevine virus A (GVA) has been reported from almost all grapevine growing regions of the world including most parts of Iran where grapes are grown (Rakhshandehroo *et al.*, 2005; Ebrahim-Qomi *et al.*, 2007). GVA has been found closely associated with Kober stem grooving, one of the four economically important disease of the rugose wood complex (Garau *et al.,* 1994) and Shiraz disease (Goszczynski and Jooste, 2003). GVA, the type species of the genus *Vitivirus* in the family *Flexiviridae* (Martelli, 1997; Adams *et al.*, 2004) is a phloem-associated virus with flexuous filamentous particles of about 800 nm in length. It contains positive-sense single-stranded RNA, comprising five slightly overlapping ORFs. The genome is capped at the 5′ terminus and polyadenylated at the 3′ end (Adams *et al.*, 2004). As the major grape growing regions in Iran are GVA infected, it is necessary to produce virus-free mother plants for controlling the virus and securing healthy propagation material.

Cryopreservation, which offers maximal stability of phenotypic and genotypic characteristics of stored germplasm, and requires minimal storage space and maintenance, is regarded as an ideal means of long-term conservation of germplasm

[1] Department of Plant pathology, Faculty of Agriculture, Tarbiat Modares University, Tehran, Islamic Republic of Iran.

* Corresponding author; e-mail: shamsbakhsh@modares.ac.ir

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(Engelmann, 1997; Wang and Perl, 2006). To date, cryopreservation has been widely applied to plant species from tropical and temperate regions (Engelmann, 1997; Wang and Perl, 2006). With the idea that cryopreservation or cryotherapy, which requires the use of shoot tips, might be considered as a possible method for combining virus elimination and germplasm conservation, Brison *et al.* (1997) were the first to successfully apply this technique for removal of plum pox virus (PPV) from an interspecific *Prunus* rootstock. Using the same method, Helliot *et al.* (2002) eliminated cucumber mosaic virus (CMV) and banana streak virus (BSV) of *Musa spp*. shoot tips and Wang *et al.* (2003) eradicated GVA from *V. vinifera* L. cv. Bruti. Until now, cryotherapy has been successfully applied to eliminate nine viruses (Wang *et al*., 2009; Wang and Valkonen, 2009), sweet potato little leaf phytoplasma (Wang and Valkonen, 2008a) and Huanglongbing bacterium causing "citrus greening" (Ding *et al.*, 2008). Cryotherapy is becoming a new biotechnological method for plant pathogen elimination (Wang *et al.,* 2009; Wang and Valkonen, 2009).

Electrotherapy has been applied to eradicate potato virus X (PVX) from different infected potato clones (Lozoya–saldana *et al.*, 1996). Applying electricity on garlic, sugarcane, potatoes and Araceae, eliminated potyvirus, luteovirus and carlavirus respectively (Hernandez *et al.*, 1997). The efficiency of electrotherapy has previously been investigated on banana streak virus (BSV)-infected banana plants and 40-80% of plants were sanitized (Hernandez *et al.*, 2002).

GVA elimination has been achieved by combination of thermotherapy and meristematic cultures (Guidoni *et al.,* 1997), meristem cultures (Bottalico *et al.,* 2000), *in vitro* somatic embryogenesis (Gambino *et al.,* 2006), cryopreservation (Wang *et al.,* 2003), chemotherapy and *in vitro* thermotherapy at 36°C for 57 days (Panattoni *et al*., 2007). The aim of the present research was to eliminate GVA from *V. vinifera* L. cv Black by cryotherapy and electrotherapy treatments and to compare their efficiency in terms of survival and virus elimination.

MATERIALS AND METHODS

Plant Material

Cuttings of infected field-grown *V. vinifera* cv. Black were collected, planted in plastic pots containing a mixture of perlite, soil, and peat (1:1:1 by volume) and kept in greenhouse conditions at 24°C±2. Their sanitary status was assayed by RT-PCR. Healthy grapevines to serve as negative control were obtained by sowing grape seeds which were first treated with 0.01 M gibbrellic acid for 24 hours.

Encapsulation, Dehydration, and Cryotherapy

The procedure was performed according to Wang *et al.* (2003), with some modifications. Shoot tips (1 mm in size) were excised from green shoots of two-three years old grapevines and suspended in a solution of ¾ Murashige and Skoog (1962) medium supplemented with 3% (w/v) Na-alginate, 2 M glycerol, 0.4 M sucrose, and 2 µM benzylaminopurine (BA). The medium and the shoot tips were transferred with a sterile pipette into a 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose at room temperature. Shoot tips were kept for 30 minutes to form beads (about 4 mm in diameter). The beads were then precultured on ¾ MS medium with increasing sucrose concentrations of 0.25, 0.5, 0.75 and 1.0 M for 4 days, one day at each concentration. The beads were then placed on sterilized filter paper in Petri dishes and dehydrated by air drying in laminar flow at room temperature for 12 hours. Following dehydration, beads were transferred into a 5 ml cryotube and immersed directly in liquid nitrogen for one hour. Then they were

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rapidly thawed in a water bath at 40ºC for 3 minutes. Thawed beads were post-cultured on a Petri dish containing solidified ¾ MS medium containing 2 µM benzylaminopurine (BA). Petri dishes were maintained in the dark at 24˚C for 2 days and, then, were maintained at a temperature of 24±2ºC under a 16-h photoperiod with a light intensity of 45 µE s¹ m² provided by cool-white fluorescent tubes. After 6 weeks, shoots longer than 3 mm were transferred to solid ¾ MS medium for regeneration of plants. All experiments were repeated twice.

Electrotherapy

Green cane pieces, three cm long and comprising a bud, were collected from the GVA infected grapevine and washed with tap water for 20 minutes. Their leaves were cut off and then exposed to electric currents of 0, 10, 20 and 30 milliamps (mA) for 10 or 15 minutes using a horizontal electrophoresis apparatus in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8) followed by immediate sterilization in 2.5% (w/v) of sodium hypochlorite for five minutes and were then washed again with sterilized water for 10 minutes. MS basal medium, modified with 1 mg l^{-1} (4.44 µM) BA, 0.5 mg l^{-1} (2.675 µM) NAA, 50 mg l^{-1} ascorbic acid, 50 mg l^{-1} acetic acid, 30 g l^{-1} sucrose and 8 g l^{-1} agar, was used for nodal explants culture. After two months, generated plantlets were tested by RT-PCR for the presence of virus.

Virus Detection by RT-PCR

Total RNA was extracted from fresh leaves according to a modified method of Dellaporta *et al*. (1983) described by Rowhani *et al*. (1993).

The PCR primers were derived from GVA-GTG11-1 sequence with the forward primers GVA-H7038 (5´-AGGTCCACGTTTGCTAAG-3´) identical to nucleotides 7038-7055 and the reverse primer GVA-C7275 (5´-CATCGTCTGAGGTTTCTA-3´) complementary to nucleotides 7257-7275 (Goszszynski *et al.,* 2008, Gene bank accession No: DQ855084.2), in a single one-tube RT-PCR reaction as described by Nassuth *et al.* (2000). The RT-PCR amplification was performed in a 25 µl reaction volume containing 2.5 µl total RNA extract, 2.5 µl 10× PCR buffer (CinnaGen, Iran) 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (dNTPs), 20 units M-MuLV (RevertAid™ M-MuLV Reverse Transcriptase, CinnaGen, Iran), 2 units *Taq* polymerase (CinnaGen, Iran), and 0.5 µM of both the complementary and homologous primers specific. Amplification was carried out in a thermocycler (Eppendorf ep Gradient, Germany) using a 60 minutes step at 42°C (reverse transcription), followed by a step at 94°C for 3 minutes and then 35 cycles of the following: 94°C for 30 s, 56°C for 45 s, and 72°C for 60 s, with a final elongation step at 72°C for 15 minutes.

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in TBE buffer followed by staining with ethidium bromide (0.5 µg ml^{-1}). DNA molecular weight markers (GeneRuler™100bp DNA ladder, Fermentas, Lithuania) were used to determine the size of the amplified fragments.

RESULTS

Effect of Different Steps during Cryotherapy on Explants Survival and GVA Elimination

To determine if GVA elimination by cryotherapy of shoot tips only occurs during the freezing step, plantlets recovered from different steps during the cryotherapy were tested for GVA. As shown in Table 1, the survival rate of the control shoot tips was 100% in all cases. After freezing in liquid nitrogen, 59% of the shoot tips survived.

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Table 1. Effect of different steps during cryotherapy of shoot tips by encapsulation-dehydration on survival and GVA elimination from grapevine cv. Black. Shoot tips used in this experiment were 1 mm in size. Data are presented as Mean±SE.

Treatment	Survival		GVA elimination plants^{-1}	
	ratio	%	ratio	%
Control[a]	6/6	100	0/6	0.0
Encapsulation	6/6	100	0/6	0.0
Dehydration	6/6	100	0/6	0.0
Freezing	19/ 32	59%±1.4	8/19	42.2%±0.8

[a] Non-treated shoot tips.

GVA detection by RT-PCR showed that all plantlets recovered from the steps before freezing were GVA-infected, indicating that none of the steps before freezing were able to eliminate GVA. In contrast, as many as 42.2 % of the plantlets recovered from freezing were GVA-free (Figure 1).

Plant Regeneration and Establishment ofPlantlets under Greenhouse Conditions

Shoot tips that survived from cryotherapy had green colour 6 weeks post culture. About 4 weeks after the survival assessment, elongated shoots longer than 3 mm were transferred to MS solid medium supplemented with 1.5 mg l^{-1} (6.66 µM) BA, 1 mg l^{-1} (5.37 µM); 50 mg citric acid; and 50 mg ascorbic acid for whole plant regeneration. Nearly 100% of the plantlets became established under the greenhouse condition.

Effect of Electrotherapy on Plant Survival and GVA Elimination

After two months, generated plants were tested for the presence of virus using RT-PCR technique. In the 10 minutes exposure experiments, 100% (control), 83% (10 mA),

Figure 1. Detection of GVA by RT-PCR assay using primers GVA-H7038 and GVA-C7275 on *V. vinifera* cv. Black plantlets regenerated from cryotherapy-treated shoot tips. Lane M, molecular weight marker (GeneRuler™ 100bp DNA ladder, Fermentas); lane PC, infected plantlets no treated; lanes 1–19; plantlets regenerated from cryotherapy-treated shoot tips of GVA-infected grapevine.

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75% (20 mA) and 75% (30 mA) of the plants were regenerated but none was virus-free. In the 15-minutes exposure time experiments, regeneration rates were 100% (control), 75% (10 mA), 62.5% (20 mA) and 62.5% (30 mA). GVA was not eradicated in the controls and after the 10 mA exposure, while plantlets 1 and 2 were, GVA-free following exposure to, respectively, 20 and 30 mA (Table 2 and Figure 2). Morphology of some regenerated plants from electrotherapy-treated shoot tips showed some abnormality (data not shown).

DISCUSSION

In the present study, 42% GVA-free plantlets were regenerated from cryotherapy-treated shoot tips of GVA-infected grapevine and their morphology was similar to that of the control shoot tips. As clearly shown in Table 1, the effect of cryotherapy on GVA elimination is associated exclusively with the freezing step; the other steps involved in the process of cryotherapy do not affect viral eradication. Wang *et al.* (2003) reported 97% virus eradication from GVA-infected *V. vinifera* cv Bruti using cryopreservation. The reasons for this difference in virus elimination efficiency are not known, although factors such as the use of shoot tips from greenhouse plants as well as the different dehydration protocol, grapevine variety, and, perhaps, virus strain may have had a bearing in determining it. Moreover, detection method also might have caused this difference. In this study, RT-PCR was applied to assess the sanitary condition of the regenerated plants, whereas Wang *et al.* (2003) used Western blot analysis and MP GAV antibody.

It has been shown by light-microscopy observation that freezing in liquid nitrogen kills the cells with large vacuoles in the lower layers of the meristem, while leaving a few top layers of dense cells alive (Brison *et al.*, 1997; Wang *et al.*, 2008; Wang and Valkonen, 2008b, Wang *et al.,* 2009; Wang and Valkonen, 2009). It is well-known that the distribution of viruses in infected plants is uneven. In fact, the meristematic dome is generally either free or contains a very low virus titre, while it increases with increasing distance from the meristem dome. Cells of the meristematic dome are characterized by their small size, a high nucleo-cytoplasm ratio and dense cytoplasm with small vacuoles (Helliot *et al.*, 2002; Wang and Valkonen, 2008a). The nucleo-cytoplasm ratios decrease progressively with increasing distance from the meristematic dome. When shoot tips are subjected to freezing in liquid nitrogen, large cells with bigger vacuoles and containing more water, which are likely to be infected by the virus, are killed. Only small cells with dense cytoplasm, which are located in the top layers of meristem and are likely to be virus-free, survive freezing in liquid nitrogen (Helliot *et al.*, 2002; Wang *et al.*, 2003; Wang and Valkonen, 2008b). This is thought to provide an explanation for why plants regenerated from cryotherapy can be freed from virus.

In this study, sixty eight pieces of 3 cm long

Table 2. Effect of electric current intensity and treatment duration on percentages of shoot regeneration and GVA elimination from grapevine cv. Black.

Treatment Minutes-mA	Survival %	GVA elimination plants^{-1}
10´- 10	83.3	0/12
10´- 20	75	0/8
10´- 30	75	0/8
10´- 0	100	0/8
15´- 10	75	0/8
15´- 20	62.5	1/8
15´- 30	62.5	2/8
15´- 0	100	0/8

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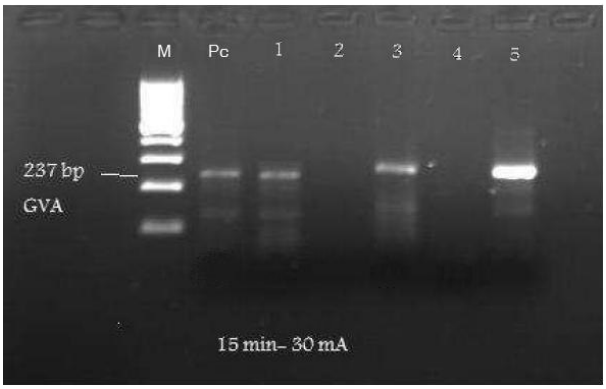

Figure 2. Detection of GVA by RT-PCR assay using primers GVA-H7038 and GVA-C7275 on *V. vinifera* cv. Black plantlets regenerated from electrotherapy-treated cane. Lane M, molecular weight marker (GeneRuler^{TM} 100bp DNA ladder, Fermentas); lane PC, infected plantlets not treated; lanes 1– 5, plantlets regenerated from electrotherapy-treated at 30 mA for 15 minutes exposure time.

cuttings were exposed to electric currents of 0, 10, 20 and 30 mA for 10 or 15 minutes followed by culture of explants. There was no virus elimination in any of the intensity treatments after 10 minutes exposure, whereas after 15 minutes exposure to 20 and 30 mA, 20% and 40% of plantlets were GVA-free, respectively. Previous studies have shown that exposing plant tissues to electricity increases the temperature inside the cells and that the extent of this increment depends on conditions such as age, volume, hydric potential, thickness of tissue and cellular constitution (González *et al.*, 2006). Denaturation of the protein moiety of virus particles may occur by increasing the temperature of the cells and may involve also normal plant proteins including components that are influential in plant growth and regeneration (González *et al.*, 2006). The result of this study indicated that the morphology of plants regenerated from electrotherapy-treated shoot tips show some abnormality. It is very likely that the temperature increase in the cells has an effect on their protein synthesis and, thus, results in abnormal growth of the regenerated plants. Therefore, it is important that the temperature reached in the buds during electric treatment stays in the safe range. Although temperature-induced denaturation of the virus coat protein is irreversible, this should not affect the infectivity, because RNA is unlikely to be affected.

What is more, the plantlets subjected to different sanitation treatments were tested for the presence of the virus only once, two to four months after the sanitation treatment. This does not provide any certainty on the actual success in elimination of GVA. Results would have been more reliable, if the treated plants were repeatedly tested to avoid an erroneous diagnosis due to the undetectable limits.

Taking into account these considerations, it can be stated that electrotherapy of grapevine shoot tips is a potential tool and cryotherapy is an efficient and convenient protocol for elimination of GVA in the infected grapevines.

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حذف ویروس A مو (GVA) با استفاده از روش های سرمادرمانی و برق‌درمانی

ش. بیاتی، م. شمس بخش و ا. معینی

چکیده

ویروس ای مو (Grapevine virus *A*, GVA) تقریباً از همه‌ی تاکستان‌های عمده ایران گزارش شده است. از آنجایکه مو به روش رویشی مانند قلمه یا پیوند زدن ازدیاد می یابد، آلودگی ویروسی از طریق پایه مادری به نسل های بعدی منتقل می شود. از این رو کنترل ویروس‌های مو از طریق تولید گیاهان مادری سالم دست یافتنی است. در تحقیق حاضر روش های سرمادرمانی و برق‌درمانی برای حذف GVA از جوانه های مو به کار گرفته شد و کارآیی آنها مقایسه گردید. میزان باززایی در روش سرمادرمانی ۵۹٪ بود. ردیابی GVA با استفاده از روش RT-PCR نشان داد که ۴۲٪ از گیاهان باززایی شده عاری از ویروس بودند. در روش برق‌درمانی، تاثیر شدت جریان و مدت تیمار روی بقای گیاه و حذف ویروس بررسی شد. بهترین نتیجه با استفاده از شدت جریان ۳۰ میلی آمپر به مدت ۱۵ دقیقه به دست آمد. تحت این شرایط ۶۲٪ گیاهان باززایی و از این میان ۴۰٪ عاری از ویروس شدند. این اولین گزارش از کاربرد موفقیت آمیز برق‌درمانی برای حذف GVA از گیاهان آلوده می باشد همچنین نتایج به دست آمده نشان داد که سرمادرمانی روشی موثر و مناسب برای حذف GVA از مو آلوده بود.

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