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Incidence of *Grapevine Fanleaf Virus* in Iran: A Survey Study and Production of Virus-Free Material Using Meristem Culture and Thermotherapy

S. A. Salami[1)], A. Ebadi[1)], Z. Zamani[1)] and M. Koohi Habibi[2)]
(Faculty of Horticultural Sciences and Plant Protection, [1)]Department of Horticulture and [2)]Department of Plant Protection, University of Tehran, Karaj, Iran)

Summary

The occurrence, geographical distribution and rate of incidence of *Grapevine fanleaf virus* (GFLV) were surveyed in 44 vineyards and nurseries in different regions of Iran. GFLV infection was confirmed by mechanical transmission onto herbaceous hosts, DAS-ELISA and IC-RT-PCR. Two thermal conditions, as well as meristem culture were used to produce virus-free material. Results confirmed the presence of GFLV in Bavanat, Shiraz, Jahrom, Maymand, Urmia, Karaj and Naghadeh. Both sanitation protocols significantly reduce the level of GFLV infection, however, a combination of meristem culture and thermotherapy produced 100 % virus-free material from the cultivars 'Bidaneh Sefid' and 'Shahroodi'.

Key words. DAS-ELISA – IC-RT-PCR – nepovirus – meristem – thermotherapy – *Vitis vinifera*

Introduction

Grapevine, an important fruit crop, is highly affected by various infectious viral diseases (Martelli and Savino 1990). Fanleaf degeneration, a disease caused by *Grapevine fanleaf virus* (GFLV), is the most destructive viral disease worldwide reducing the yield by up to 80 %. GFLV is though to have originated from ancient Persia and then spread to the west through infected propagation material (Vuittenz 1970; Zaki-Aghl and Izadpanah 2003). The first report of GFLV in Iran was based on visual symptoms (Vuittenz 1970). The presence of GFLV in Urmia, North-west of Iran, was later reported by Ghorbani 1988. Recently, reports concerned the incidence and prevalent of GFLV in Iranian vineyards (Rakhshandehroo et al. 2005; Bashir and Hajizadeh 2007). Control of viruses is basically based on preventive measures such as selection, sanitation, and breeding for resistance. Considerable progresses were made regarding the implementation of certification schemes in several countries, most, focused on using certified propagation material obtained from *in vitro* meristem culture and heat therapy (Gifford and Hewitt 1961; Lehoczky et al. 1992; Szegedi 1995; Leonhardt et al. 1998). Due to the severity and wide distribution of GFLV and other viral diseases in Iran (Habili et al. 2003; Rakhshandehroo et al. 2005), the increasing demand for sustainable and environmentally safe viticulture practices and intensive sanitation program, there is a great need to develop appropriate experiments in order to evaluate the effect of the meristem culture and thermotherapy for GFLV eradication and also to set up systems for selecting material free of viruses. The goal of the current study was to determine the incidence and distribution of GFLV in Iran as well as the ability of meristem culture and thermotherapy in virus-free plant material production.

Materials and Methods

Plant Material

GFLV infected cuttings of *Vitis vinifera* cultivars 'Bidaneh Sefid' and 'Shahroodi' were used as plant material. A survey study was conducted by screening 44 vineyards and nurseries located in different regions of Iran (Table 1), and a total of 882 canes with typical symptoms of GFLV infection (Raski et al. 1983) were collected during mid and late summer and tested for infection. In order to obtain further infected materials for meristem culture and thermotherapy studies, green grafting was used to infect cuttings due to the high virus load that is delivered into the vascular by this method (Taylor et al. 1967; Lahogue et al. 1995).

Detection of GFLV

GFLV infection was confirmed by mechanical transmission onto herbaceous hosts, serological and molecular approaches. *Chenopodium amaranticolor*, *Chenopodium quinoa*, and *Gomphrena globosa* at the 4-leaf stage (two well expanded and two young apical leaves) were inoculated with leaves extract of candidate plants. Inoculation buffer used was 0.1 M phosphate buffer, pH 7.2. One gram of leaf tissue was ground in 5 mL of buffer in a sterile mortar and the sap extract rubbed on carborun-

dum-dusted leaves. Control plants were mock-inoculated with inoculation buffer. The inoculated plants were grown in a glasshouse at 22–24 °C under 50–60 % relative humidity and with a 16 h photoperiod. Double antibody sandwich- (DAS-) ELISA with an anti-GFLV antibody was performed in microtiter plates as described by CLARK and ADAMS (1977). Samples were tested in duplicate. Absorbance was determined at 405 nm. The samples with optical densities (ODs) that were of at least twice as high as the ODs measured in the negative control were considered infected. Immunocapture (IC) reverse-transcriptase (RT-PCR) was used based on the description of WETZEL et al. (1992). A 25 μL PCR mix contained 1.5 mM $MgCl_2$, 1 pmol CP433V and CP912C primers (IZADPANAH et al. 2003), 1 × PCR buffer, 200 μM dNTPs, 1 U *Taq* DNA Polymerase (Fermentas) and 5 μL of cDNA. PCR with was performed with 1 cycle of 94 °C for 2 min, 35 cycles of 94 °C for 1 min, followed by 45 s at 51 °C and finally 45 s at 72 °C. A final extension step was for 10 min at 72 °C. The PCR products were separated on 1.2 % agarose TAE gel containing ethidium bromide.

Meristem culture and thermotherapy treatment

Shoot tips were surface sterilized in 70 % ethanol for 30 s and subsequently soaked for 3 min in 0.1 % aqueous solution of AgCl, plus a few drops of Tween-20 with constant stirring and finally were rinsed three times in sterile distilled water. Outer leaves were removed and meristem tips (0.3–0.5 mm in length) were dissected under a stereomicroscope. Meristems were established on MURASHIGE and SKOOG (1962) basal medium supplemented with 0.5 mg L^{-1} BA for 'Bidaneh Sefid', and 1.0 mg L^{-1} BA+0.5 mg L^{-1} IBA for 'Shahroodi', 7 g L^{-1} agar and 30 g L^{-1} of saccharose and incubated in a growth chamber at 28/24 °C (16 h/8 h light-dark) with a light intensity of 40 μE m^{-2} s^{-1} provided by white fluorescent tubes. Newly formed shoots of more than 1 cm in length were transferred to fresh proliferation medium containing the same medium but different combinations of plant growth regulators included BA (0.5, 1.0 and 1.5 mg L^{-1}) in alone and/or in combination with IBA (0,0.1 and 0.5 mg L^{-1}). The same basal medium with half strength macroelements, 20 g L^{-1} agar and 30 g L^{-1} of saccharose containing four different concentration of IBA (0.0, 0.05, 0.2 and 0.5 mg L^{-1}) was used for rooting shoots. Rooted plants were then transferred to Jiffy pots containing Perlite and Peatmoss (1:1 v/v). Plantlets were maintained under a continuously misting system in a glasshouse for 10 d. To acclimatize the plants, the relative humidity was gradually decreased to attain complete acclimatization and active growth in the new *extra vitrum* conditions.

Individual GFLV-infected cuttings were heat treated during seven weeks under the following conditions: (1) Control: infected plants were maintained in a controlled glasshouse at 22±2 °C. (2) Heat treatment: Infected plants were placed in a thermostatically controlled chamber at 40/30 °C or 30/25 °C day/night temperature with the selected photoperiod (16 h/8 h light-dark). After seven weeks thermotherapy, apical meristem from plants treated at both 40/30 °C and 30/25 °C were excised and grown into plantlets *in vitro*.

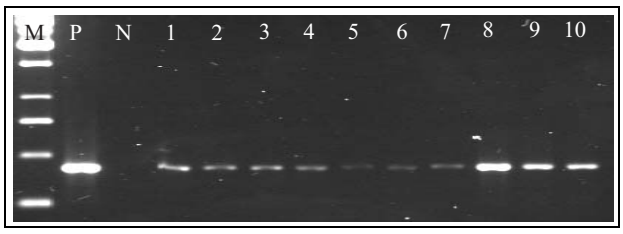


Fig. 1. Amplifications of the expected 480 nt. DNA fragments resulted from immunocapture (IC) reverse-transcriptase (RT-PCR) with *Grapevine fanleaf virus* (GFLV)-specific primer pair CP433V/912C. P: Positive control; N: Negative control (healthy grapevine); Lanes 1–10 are infected samples; M: 1 Kb DNA ladder.

Results

Detection of GFLV

Results from inoculation onto herbaceous hosts and DAS-ELISA confirmed the GFLV infection in new regions, e.g. some nurseries located in Karaj and also in Naghadeh. IC-RT-PCR was used as a highly sensitive and reproducible system for virus detection (WETZEL et al. 1992) and amplifications of the expected 480 nt. DNA fragments resulted from PCR with CP433V and CP912C primers (Fig. 1). The most common symptoms observed on infected vines among 882 samples were mosaic form and the opening of petiolar sinuses in leaves (RAKHSHANDEHROO et al. 2005). Inoculation of GFLV into young plantlets of *C. amaranticolor* led to symptom development within 8–10 d (VALAT et al. 2003); on most of plants yellowish discoloration and necrotic local lesions were visible. Systemically infected leaves also showed mottling and deformation. In *C. quinoa* systemic vein clearing, leaf distortion and chlorotic spotting were appeared 12 d after inoculation with GFLV-positive extract. In *G. globosa* chlorotic local lesions appeared within 7–8 d and soon turned reddish. The average absorbance from DAS-ELISA analysis in infected samples was 2.6 fold more than healthy samples. Among 882 suspected field samples, 204 were confirmed to be infected by GFLV. The rate of incidence varied from 0.0 to 63.7 %. In accordance with ZAKI-AGHL and IZADPANAH (2003) the most infectious plants were observed in Bavanat (Table 1).

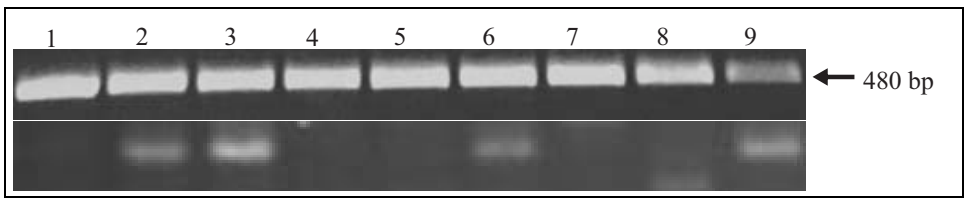


Fig. 2. Infected plants in which the GFLV had been eliminated due to high glasshouse temperature.

Table 1. Regions and number of samples tested for *Grapevine fanleaf virus* (GFLV) infection in Iran.

Regions	Cultivar	No. of nurseries (N) and/or vineyards (V) were surveyed	*Grapevine fanleaf virus* (GFLV)	
			No. of positive samples/ tested samples	Infection rate (%)
Karaj	'Bidaneh Sefid'	4 N+2 V	2/ 110	1.8
Bavanat	Unknown	5 V	65/ 102	63.7
Urmia	'Bidaneh Sefid'	7 V	53/ 123	43.1
Naghadeh	'Bidaneh Sefid'	3 V	8/ 96	8.3
Abhar	'Bidaneh Sefid'	7 V	0/ 130	0.0
Jahrom	Unknown	5 V	30/ 117	25.6
Shiraz	Unknown	3 N+3 V	27/ 105	25.7
Maymand	Unknown	5 V	19/ 99	19.2

Eradication of GFLV by meristem culture and Thermotherapy

In vitro meristem tips from GFLV-infected stock plants were used to eradicate the virus. Meristems established well in MS media supplemented with 0.5 mg L^{-1} BA for 'Bidaneh Sefid' and 1.0 mg L^{-1} BA+0.5 mg L^{-1} IBA for 'Shahroodi'. Media containing 1.5 mg L^{-1} BA for 'Bidaneh Sefid' and 1.5 mg L^{-1} BA+0.1 mg L^{-1} IBA for 'Shahroodi' induced the best results at the proliferation stage (Table 2). Adventitious shoots appeared three weeks after the first sub-culture. The MS/2 medium containing 20 g L^{-1} saccharose without IBA is a suitable medium for rooting of grapevine explants, 'Shahroodi' and 'Bidaneh Sefid' (data not shown). Of the plants generated in tissue culture, 90 % were successfully transferred to the glasshouse and subsequently tested for infection. Table 3 shows that 80 % of both cultivars derived from meristems were found to be virus-free.

Testing of plants that had been subjected to thermotherapy indicated that 70 % of plants of 'Bidaneh Sefid' and 90 % of plants of 'Shahroodi' treated at 40/30 °C for 7 weeks were free of GFLV. In contrast only 50 % of plants were virus-free following treatment at 30/25 °C (Table 3). Plants which had undergone the 40/30 °C treatment displayed some damage after 7 weeks. Unexpectedly, some infected grapevines within the glasshouse which had not undergone any thermotherapy treatment and were kept as control, were found to be virus-free (Fig. 2). This observation led us to the assumption that a form of natural heat therapy had occurred in glasshouse during June and July when day temperatures reached 35–40 °C (Valero et al. 2003).

Heat therapy combined with meristem culture eradicated GFLV from infected plants by 100 % (Table 3). There were no significant differences between the two cultivars studied when thermotherapy and meristem culture were used together. Finally, virus-free plants were multiplicities through an efficient technique using fragmented shoot apices (Barlass and Skene 1978; Salami et al. 2005).

Discussion

In spite of a wide range of grapevine varieties are cultivated in Iran, and survey reports have been determined the presence of viruses (Rakhshandehroo et al. 2005; Bashir and Hajizadeh 2007), no comprehensive sanitary programs developed to ensure virus-free material production in Iran yet. Virus-infected vines are normally propagated using cuttings, a process that perpetuates infections. As a first step towards establishing a certification scheme, we have focused on GFLV because it is widespread in other parts of the world and causes severe disease. We also attempted to developed an optimized sanitation protocols significantly reduced the level of GFLV infection. In this study the 100 % sanitation obtained by combining *in vitro* meristem culture and heat therapy. By combining these two methods several authors have succeeded in the eradication of GFLV (Gifford and Hewitt 1961; Barlass et al. 1982). The occurrence of GFLV had also been briefly reported in some area of Iran and confirmed in new sites e.g. Karaj and Naghadeh in this survey study. Despite of previous report on GFLV incidence in Abhar (Rakhshandehroo et al. 2005), all cuttings were gathered from vineyards in this region were GFLV-free. However, in order to determine the precise incidence level of GFLV in Iran need to carry out more extensive surveys with a larger number of samples and in several consecutive years.

Wide distribution of GFLV in distinct parts of Iran and absence of ectoparasitic dagger nematode *Xiphinema index* from these regions, presume that infection of individual plants might result from using either infected cuttings or scions. This might confirm that infected material were the main components for vast distribution of this virus to the new geographical sites (Vuittenz 1970). Due to the severity of viral epidemics, the difficulties of implementing efficient control strategies, and the increasing demand for sustainable and environmentally-safe viticulture practices, there is a great need to develop techniques capable of producing virus-free propagation material. These techniques included micro-grafting (Engelbrecht and Schwerdtfeger 1979), *in vitro* culture of meristems or shoot tips (Barlass et al. 1982; Maekawa et al. 1995; Gok and Ergenoglu 1997), heat therapy (Monette 1986) and recently, somatic embryogenesis (Gambino et al. 2008) reduce the infection rate, however, generally the best results have been obtained when these methods are combined with heat treatment (Gifford and Hewitt 1961; Postman and Hadidi 1995; Buciumeanu and Visoiu 2000). Mersitem culture takes advantage that some viruses are unable to infect this region because of inhibition of replication and restriction of virus movement (Faccioli and Marani 1998). The effect of heat on viruses is not well

Table 2. Effect of different plant growth regulators on proliferation of grapevine cultivars 'Bidaneh Sefid' and 'Shahroodi' (Means separation within columns by Duncan's Multiple Range Test, P=0.05).

Final status of plantlets	Leaf status	Mean length of shoot (cm)	Mean number of shoot	Cultivar+	BA (mg L^{-1})	IBA (mg L^{-1})
1.7 j	1.2 g	2.8 de	1.0 c	BS	0.5	0.0
2.2 ij	1.4 fg	2.9 de	1.6 abc	BS	0.5	0.1
1.9 j	1.8 efg	3.0 cd	1.2 bc	BS	0.5	0.5
2.0 j	1.4 fg	3.3 cb	1.6 abc	SH	0.5	0.0
1.8 j	1.6 fg	3.4 b	1.2 bc	SH	0.5	0.1
1.9 j	1.6 fg	3.8 a	1.8 abc	SH	0.5	0.5
3.2 def	2.6 cd	2.4 fgh	1.8 abc	BS	1.0	0.0
2.8 efg	2.4 cde	2.3 gh	1.8 abc	BS	1.0	0.1
2.1 ij	2.0 def	2.4 fgh	1.4 abc	BS	1.0	0.5
3.3 cde	3.0 abc	2.7 def	2.0 abc	SH	1.0	0.0
3.6 cd	2.6 cd	2.7 def	1.4 abc	SH	1.0	0.1
2.6 ghi	2.4 cde	3.0 cd	2.2 ab	SH	1.0	0.5
4.4 a	3.4 ab	1.9 i	2.2 ab	BS	1.5	0.0
4.3 a	2.8 bc	2.2 hi	2.2 ab	BS	1.5	0.1
2.3 ghij	2.0 def	2.4 fgh	1.4 abc	BS	1.5	0.5
3.8 bc	3.4 ab	2.3 gh	2.0 abc	SH	1.5	0.0
4.6 a	3.6 a	2.6 efg	2.4 a	SH	1.5	0.1
2.7 fgh	2.4 cde	2.7 def	2.2 ab	SH	1.5	0.5

+ BS = 'Bidaneh Sefid'; SH = 'Shahroodi'

Table 3. Percentage of GFLV-free plants were obtained after sanitation.

Cultivar+	Thermotherapy (40/30°C) Duration (in weeks)				Thermotherapy (30/25°C) Duration (in weeks)			
	4	5	6	7	4	5	6	7
BS	0%	20%	60%	70%	0%	0%	30%	50%
SH	0%	30%	80%	90%	0%	10%	30%	50%

	Meristem culture	Thermotherapy+ Meristem culture
BS	80%	100%
SH	80%	100%

+ BS = 'Bidaneh Sefid'; SH = 'Shahroodi'

understood but it is believed to be effective in inhibiting viral replication and synthesis of movement proteins mainly by blocking transcription (Mink et al. 1998). Metabolic changes in plants that are forced to undergo rapid growth rather than alternation in virus during thermotherapy may also be a cause of a reduced virus invasion of the apical explants (Valero at al. 2003).

In conclusion, GFLV is geographically widespread in Iran. It needs to survey the vineyards for presence of other viruses and improve the sanitary programs. In present study we have optimized two sanitation protocols for GFLV eradication that could be definitely used as powerful techniques for eradication of other plant viruses.

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Addresses of authors: Seyed Alireza Salami (corresponding author), Ali Ebadi, and Zabiholah Zamani, Faculty of Horticultural Science and Plant Protection, Department of Horticulture, University of Tehran, Karaj, Iran, and Mina Koohi Habibi, Faculty of Horticultural Science and Plant Protection,Department of Plant Protection, University of Tehran, Karaj, Iran, e-mail: asalami@ut.ac.ir