

ATTEMPTS TO ELIMINATE *GRAPEVINE RUPESTRIS STEM PITTING-ASSOCIATED VIRUS* FROM GRAPEVINE CLONES

I. Gribaudo, G. Gambino, D. Cuzzo and F. Mannini

Plant Virology Institute of the CNR (IVV-CNR), Grugliasco Unit, Via L. da Vinci 44,
10095 Grugliasco (TO), Italy

SUMMARY

The presence of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) together with other viruses can cause severe damage to infected grapevine plants, therefore the use of GRSPaV-free vines for multiplication and vineyard planting is highly desirable. This paper reports on several virus eradication techniques applied to grapevine clones in attempts to eliminate GRSPaV. The presence of GRSPaV was determined by RT-PCR. The traditional sanitation techniques, i.e. meristem tip culture, *in vivo* and *in vitro* thermotherapy, did not provide satisfactory results since more than two-thirds of derived lines were still GRSPaV-infected after treatment. However, regeneration through somatic embryogenesis always gave rise to plants free from GRSPaV. Among the tested techniques, somatic embryogenesis seems to be the most promising procedure for GRSPaV elimination, despite the technical problems related to the use of this technique and the theoretical possibility of somaclonal variation.

Key words: GRSPaV, meristem tip culture, somatic embryogenesis, thermotherapy, *Vitis*.

INTRODUCTION

The rugose wood (RW) complex is a widespread viral disease of grapevine (*Vitis* spp.), characterized by alterations of the plant woody cylinder. Four distinct RW syndromes can be identified by graft inoculation to indicator vines: *Rupestris stem pitting* (RSP), *Kober stem grooving*, *LN33 stem grooving* and *corky bark* (Martelli, 1993). RW affects the movement of water and nutrients through the vascular system and may cause graft incompatibility, delayed budburst, reduction of vigour and yield, severe decline and, in hot climates, even death of grapevines (Credi and Babini, 1996; Bonfiglioli *et al.*, 1998; Mannini, 2003).

RSP appears to be the most common disease of the RW complex (Meng *et al.*, 1999a). For many years the aetiology of the RSP disease was not known, but much progress was made in the last decade. *Grapevine rupestris stem pitting-associated virus* (GRSPaV) was identified and classified in genus *Foveavirus* (Martelli and Jelkmann, 1998; Zang *et al.*, 1998; Adams *et al.*, 2005), and the molecular variability of its genome was studied (Meng *et al.*, 1999b, 2005; Minafra and Boscia, 2003). No natural vector of RSPaV is known, but the presence of the virus in the pollen of infected vines and its transmission through seeds have been experimentally demonstrated (Rowhani *et al.*, 2000; Lima *et al.*, 2006).

GRSPaV alone induces few or no RW symptoms and some of its strains are latent in *Vitis rupestris* (Meng *et al.*, 2005). The presence of other viruses, e.g. *Grapevine virus A* (GVA), may be required for RW symptoms to occur (Bonfiglioli *et al.*, 1998). This means that cultivating symptomless but GRSPaV-infected vines carries a risk of the development of RSP if other viruses are transmitted via grafting or vectors. A close association has been recently observed between the presence of GRSPaV and symptoms of vein necrosis, suggesting that this virus may be implicated in the aetiology of this disease rather than an unknown agent as currently thought (Bouyahia *et al.*, 2005). Also, RW damage is more evident in warm climates than in cool environments with consequent problems in the exchange of propagation materials. Therefore the absence of GRSPaV in vines used for multiplication and vineyard planting is highly advisable.

The recent application of molecular tools has led to great improvements in RSP detection, but less is known about the efficacy of the various sanitation techniques used for GRSPaV eradication. While meristem tip culture is particularly effective in eliminating phloem-limited viruses, thermotherapy is normally required for the elimination of other viruses such as nepoviruses. Here we report our experiences in GRSPaV elimination performed during the procedures of grapevine clonal selection. We compare results of traditional virus eradication techniques, i.e. meristem culture and thermotherapy, and of somatic embryogenesis, which proved to be highly effective in eradicating phloem-limited viruses (Goussard *et al.*, 1991; Popescu *et al.*, 2003; Gambino *et al.*, 2006).

MATERIALS AND METHODS

Plant material and sanitation techniques. The sanitation techniques were applied to infected grapevine clones during routine genetic and sanitary selection over more than two years. Eradication of GRSPaV was attempted from a total of 26 clones of 16 wine grape (*Vitis vinifera* L.) cultivars, all from north-western Italy. The clones were infected by GRSPaV alone or were in mixed infection with other viruses. The numbers of replications for each clone and technique varied according to the availability of plant material. When possible, different sanitation techniques were applied to the same clone or cultivar. Details of cultivars treated and of the adopted sanitation technique are given in Tables 1 to 4.

Meristem tip culture. Woody cuttings were collected in vineyards during winter, stored at 4°C and forced to sprout in water at room temperature. Meristem tips (about 0.7 mm) were isolated from surface-sterilized apical buds and were cultivated on a modified Murashige and Skoog (1962) medium with half strength mineral salts and 20 g l⁻¹ sucrose (MMS) containing 9 µM benzyl-amino-purine (BAP).

In vivo thermotherapy. Two-year-old, greenhouse-grown potted plants were grown in a growth chamber at 24°C with a 16 h photoperiod and a total energy of 50 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps. After one month, the temperature in the chamber was raised to 38°C. The heat-therapy lasted from 40 days to a maximum of 95 days, being stopped when the plants showed marked signs of stress. At the end of the therapy, the apical bud (about 5 mm) and the 4-5 axillary buds closest to the apex were excised, surface-sterilized and cultivated on MMS medium containing 3 µM BAP.

In vitro thermotherapy. One month-old rooted plantlets growing on MMS medium without plant growth regulators (PGR) were heat-treated in a culture room at 34°C with a 16 h photoperiod and a total energy of 40 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps. The therapy lasted from 43 days to a maximum of 115 days, being stopped when the plants showed signs of stress (Fig. 1a). At the end of the therapy, the apical bud (about 2 mm) and the 2-3 axillary buds closest to the apex were excised and cultivated on MMS medium containing 3 µM BAP.

Somatic embryogenesis. Immature anthers and ovaries were isolated from inflorescences collected in vineyards and indirect somatic embryogenesis was induced as previously described (Gribaudo *et al.*, 2004). In brief, floral explants were cultivated on a callus induction medium containing 4.5 µM 2,4-dichlorophenoxyacetic acid and 8.9 µM BAP. After three months, calli were transferred on an embryo differentiation medium containing 10 µM 2-naphthoxyacetic acid, 1 µM BAP, 20 µM indole-3-acetic acid, and 0.25% activated charcoal.

Shoots from meristem culture (Fig. 1b) and thermotherapy were rooted by culturing for 7 days on a rooting medium (MMS containing 2.5 µM indole-butyric acid and 2.5 µM α-naphthalene-acetic acid) and subsequently on a MMS medium without PGR. Somatic embryos were isolated from the embryogenic callus and transferred to a medium (MMS but with mineral salts of Nitsch and Nitsch, 1969) without PGR for further growth (Fig. 1c) and germination. Single plantlets, derived from the various sanitation techniques, were micropropagated by repeatedly subculturing apical cuttings (3-4 cm long) on MMS medium without PGR, thus giving rise to individual lines.

Virus detection. The presence of GRSPaV in mother plants and lines obtained from the sanitation techniques was assayed by RT-PCR. Total RNA (200 mg) was extracted from mother plants (phloem scraped from mature canes collected in winter) or from *in vitro*-cultured plantlets. RNA was also extracted from phloem of 35 randomly selected plants, derived from the various sanitation techniques, after at least one-year growth in a greenhouse. All samples were immediately frozen and



Fig. 1. Application of sanitation techniques to grapevine: plantlet at the end of *in vitro* thermotherapy (a); shoot originated from meristem culture (b); somatic embryos at various developmental stages (c).

homogenized in liquid nitrogen. RNA was extracted as previously described (Gambino *et al.*, 2006). In brief, extraction buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% PVP-40, 2% sarkosyl and, added just before use, 1% 2-mercaptoethanol) was added to the homogenised sample and, after two extractions with chloroform/isoamyl alcohol (24:1 v/v), nucleic acids were precipitated by adding cold isopropanol. The pellet was resuspended in diethyl pyrocarbonate-treated water (DEPC-water) and 0.5 vol. of 6 M LiCl was added. The mixture was incubated overnight at 4°C. RNA was selectively pelleted after centrifugation and resuspension in DEPC-water.

First-strand cDNA synthesis was performed using 500 ng of total RNA treated with DNase (Sigma-Aldrich, St. Louis, MO, USA), 100 units of recombinant Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA), 50 units of RNase inhibitor (RNase out; Invitrogen Life Technologies, Carlsbad, CA, USA), 0.5 mM of dNTPs, and 2.5 µM of random nonamers. The mix for reverse transcriptase (10 µl) was incubated for 50 min at 37°C.

A set of specific primers (Primer 13 5'-GATGAG-GTCCAGTTGTTTCC-3' and Primer 14 5'-ATC-CAAAGGACCTTTTGACC-3') designed by Meng *et al.* (1999a) was used for PCR amplification. The two primers generate a fragment of 339 bp. The PCR reaction mix (20 µl) contained 1 µl of cDNA, 0.2 mM of dNTPs, 0.25 µM of each primer, 1.5 mM of MgCl₂ and 0.5 unit of Taq polymerase (PlatinumTaq polymerase; Invitrogen Life Technologies, Carlsbad, CA, USA). PCR was performed for 39 cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min. For each sample, a control amplification of *Vitis* 18S rRNA was performed using specific primers (18S-H325 5'-AAACGGCTACCACATCCAAG-3' and 18S-C997 5'-GCGGAGTCCTAAAAGCAACA-3') (Gambino *et al.*, 2006), in order to check for possible RT-PCR inhibitors or degradation of RNA. The two primers generated a fragment of 673 bp. Reaction products were analysed in 1 % agarose gels buffered in TBE (45 mM Tris-borate, 1 mM EDTA) and visualised by UV-light after staining with ethidium bromide.

Table 1. Elimination of GRSPaV from grapevine by meristem culture: cultivars tested, total number of lines obtained and number of lines found to be virus-free in RT-PCR assays.

Cultivar	Lines obtained (No.)	Lines cleaned (No.)
Albarola	7	1
Bianchetta genovese	3	0
Bizzarria	2	2
Bosco	11	8
Picabon	2	0
Rossese	13	0

All individual lines obtained were tested at least once with the above described primers.

Additionally, a second primer set (primer 9 5'-GGC-CAAGGTTTCAGTTTG-3' and primer 10 5'-ACACCT-GCTGTGAAAGC-3' designed by Meng *et al.*, 1999a), that generate a fragment of 498 bp, was also used for PCR amplification on a limited number of samples (125 lines).

Percentage data from the experiments were arcsin transformed and subjected to analysis of variance (SAS statistical software, version 8.2, SAS Institute, Cary, NC).

RESULTS

In our protocol, set up for routine clonal selection, the length of the thermotherapy (*in vivo* as well as *in vitro*) was not fixed but differed according to the health conditions of the treated plants: the heat-treatment lasted as long as possible, with an average of 59 days for the *in vivo* thermotherapy and 58 days for the *in vitro* thermotherapy. As stress often induced degeneration of the shoot apex, the number of apical buds available for excision at the end of the thermotherapy period was low, particularly after *in vitro* thermotherapy. A prolonged duration of heat therapy did not ameliorate sanitation rates. Some cultivars gave better results than others but the low number of lines obtained did not allow us to draw conclusions about the influence of genotype.

The four sanitation techniques showed different efficiencies in eradicating GRSPaV. The number of individual lines obtained for each sanitation techniques and the number of lines cleaned from GRSPaV according to the results of RT-PCR assays are reported in Tables 1 to 4.

Table 2. Elimination of GRSPaV from grapevine by *in vivo* thermotherapy: cultivars tested, therapy length, total number of lines obtained and number of lines found to be virus-free in RT-PCR assays.

Cultivar	Heat-therapy duration (days)	Lines obtained (No.)	Lines cleaned (No.)
Albarola	40	12	0
Albarola	76	4	0
Albarola	95	7	0
Bianchetta genovese	62	10	0
Bosco	40	23	10
Bosco	95	7	0
Pigato	95	3	3
Rossese	40	4	3
Vermentino	40	1	0
Vermentino	95	1	1

Table 3. Elimination of GRSPaV from grapevine by *in vitro* thermotherapy: cultivars tested, therapy length, total number of lines obtained and number of lines found to be virus-free in RT-PCR assays.

Cultivar	Heat-therapy duration (days)	Lines obtained (No.)	Lines cleaned (No.)
Barbera	47	5	0
Barbera bianca	57	7	1
Bussanello	43	4	0
Moscato	43	13	0
Nebbiolo Prunent	60	9	2
Picabon	87	2	0
Picabon	115	4	1

Table 4. Elimination of GRSPaV from grapevine by somatic embryogenesis: cultivars tested, total number of lines obtained and number of lines found to be virus-free in RT-PCR assays.

Cultivar	Lines obtained (No.)	Lines cleaned (No.)
Albarola	11	11
Bosco	2	2
Brachetto	10	10
Grignolino	35	35
Müller Thurgau	33	33
Rossese	2	2
Vermentino	4	4

The overall rates of GRSPaV eradication are reported in Table 5. On average, traditional sanitation techniques (meristem tip culture and thermotherapy) resulted in low percentages of GRSPaV eradication: more than two-thirds of derived lines remained infected, while regeneration through somatic embryogenesis always gave rise to GRSPaV-free plants. No discrepancy was observed between results of RT-PCR assays when a second primer pair for GRSPaV detection was used. Additional RT-PCR analyses, performed on 35 plants after at least one year growth in the greenhouse, confirmed the results of the previous assays on *in vitro* plantlets. An example of agarose gel analysis of RT-PCR assays with primers specific for GRSPaV (and for *Vitis* 18S rRNA as a control for RNA amplification) is shown in Fig. 2.

DISCUSSION

Although virus eradication is often performed for grapevine clonal selection and for research aims, percentages of eradication are seldom reported in scientific literature, particularly in recent years. Reasons could be (i) meristem culture and thermotherapy are now considered more routine than experimental techniques, and (ii) results can be quite variable depending on the number and type of viruses, starting material, skill of personnel and other factors.

GRSPaV was found in RSP-affected grapevines and characterized only a few years ago. Our results and the few published data indicate that the GRSPaV is particularly difficult to eliminate. GRSPaV is reported to be quite recalcitrant to elimination by meristem tip culture (Minafra and Boscia, 2003), compared to closteroviruses. G. Bottalico (University of Bari, Italy; personal communication) obtained callus formation and organogenesis from grapevine stem tissues but the regenerated shoots remained GRSPaV-infected. Even the culture of fragmented shoot apices, which proved effective in eradicating leafroll disease, could not consistently remove GRSPaV from all the grapevine cultivars tested (Habibi *et al.*, 1992).

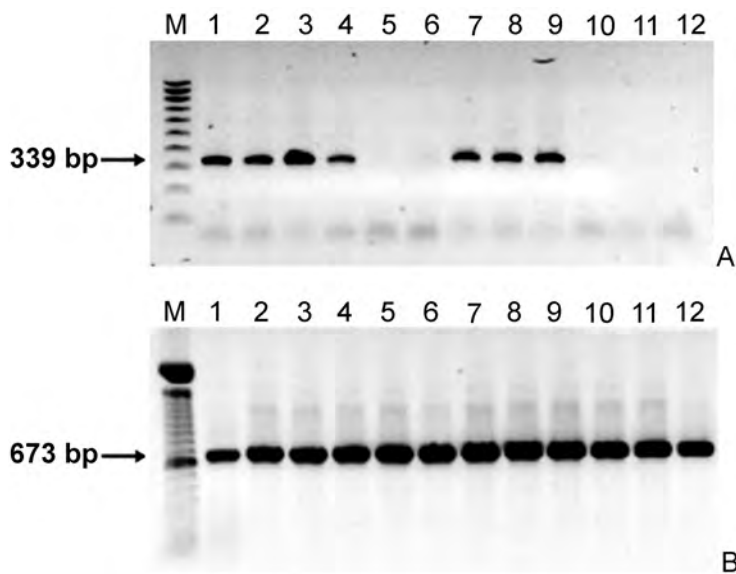
**Fig. 2.** Agarose gel analyses of RT-PCR assays with primers specific for GRSPaV (**A**) and 18S rRNA (**B**), performed on *in vitro*-cultured grapevine plantlets. Lane 1: GRSPaV positive control; lanes 2-6: lines originated from meristem culture; lanes 7-12: lines originated from thermotherapy; M, molecular weight markers.

Table 5. Elimination of GRSPaV from grapevine clones (average percentages) by various sanitation techniques. Values followed by the same letter do not differ significantly at $P=0.01$.

Sanitation technique	Total number of lines obtained	RSPaV eradication (%)
Meristem tip culture	38	28.9 B
<i>In vivo</i> thermotherapy	72	23.6 BC
<i>In vitro</i> thermotherapy	44	9.1 C
Somatic embryogenesis	97	100 A

In our laboratory, in a 13 year-period the success of virus eradication from grapevine clones by thermotherapy or meristem tip culture varied between 75% and 97% when infecting viruses were GVA, *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 3* (GLRaV-3), or *Grapevine fleck virus* (GFkV) (unpublished information). In the present study, these techniques proved to be inefficient for GRSPaV eradication. Somatic embryogenesis, which gave very good results for eradication of GLRaV-1, GLRaV-3, GVA and GFkV from several *V. vinifera* cultivars (Goussard *et al.*, 1991; Popescu *et al.*, 2003, Gambino *et al.*, 2006, and unpublished information), seems to be the most promising technique for GRSPaV elimination although it is more difficult technically, more time consuming and more cultivar dependent than the other sanitation procedures. Further tests with different grape cultivars and/or viral infections will add information about its efficacy.

The possibility of somaclonal variation has also to be taken in account and should be carefully ascertained in regenerated plantlets. With regard to this, an experimental vineyard was planted in spring 2005 in Guarene (northern Italy) with plants of cv. Grignolino cleaned through somatic embryogenesis. This vineyard will be enlarged over the next few years so that we will be able to study embryo-derived plants of several grape cultivars.

Other virus elimination techniques have been proposed, although they are less used than meristem tip culture and thermotherapy. Cryopreservation of *in vitro*-grown grape shoot tips resulted in high percentages of GVA elimination (Wang *et al.*, 2003) and new antiviral compounds have been recently tested for GLRaV-3 eradication (Panattoni *et al.*, 2006). Both techniques should be applied to GRSPaV-infected grapes and the results will help in defining the most suitable strategy for the elimination of this virus.

REFERENCES

- Adams M.J., Accotto G.P., Agranovsky A.A., Bar-Joseph M., Boscia D., Brunt A.A., Candresse T., Coutts R.H.A., Dolya V.V., Falk B.W., Foster G.D., Gonsalves D., Jelkmann W., Karasev A., Martelli G.P., Mawassi M., Milne R.G., Minafra A., Rowhani A., Vetten H.J., Vishnichenko V.K., Wisler G.C., Yoshikawa N., Zavrjev S.K., 2005. Genus *Foveavirus*. In: Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (eds.). *Virus Taxonomy, Classification and Nomenclature of Viruses*, 8th Report of the ICTV, pp. 1107-1109. Elsevier Academic Press, Oxford, UK.
- Bonfiglioli R.G., Habili N., Green M., Schliefer L.F., Symons R.H., 1998. The hidden problem – Rugose wood associated viruses in Australian viticulture. *Australian Grapegrower and Winemaker* 420: 9-13.
- Bouyahia H., Boscia D., Savino V., La Notte P., Pirolo C., Castellano M.A., Minafra A., Martelli G.P., 2005. Grapevine rupestris stem pitting-associated virus is linked with grapevine vein necrosis. *Vitis* 4: 133-137.
- Credi R., Babini A.R., 1996. Effect of virus and virus-like infections on the growth of grapevine rootstocks. *Advances in Horticultural Science* 10: 95-98.
- Gambino G., Bondaz J., Gribaudo I., 2006. Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. *European Journal of Plant Pathology* 114: 397-404.
- Goussard P.G., Wiid J., Kasdorf G.G.F., 1991. The effectiveness of *in vitro* somatic embryogenesis in eliminating fan-leaf virus and leafroll associated viruses from grapevines. *South African Journal of Enology and Viticulture* 12: 77-81.
- Gribaudo I., Gambino G., Vallania R., 2004. Somatic embryogenesis from grapevine anthers: The optimal developmental stage for collecting explants. *American Journal of Enology and Viticulture* 55: 427-430.
- Habili N., Krake L.R., Barlass M., Rezaian M.A., 1992. Evaluation of biological indexing and dsRNA analysis in grapevine virus elimination. *Annals of Applied Biology* 121: 277-283.
- Lima M.F., Rosa C., Golino D.A., Rowhani A., 2006. Detection of Rupestris stem pitting associated virus in seedlings of virus-infected maternal grapevine plants. *Extended Abstracts 15th Meeting of ICVG, Stellenbosch* 2006, 244-245.
- Mannini F., 2003. Virus elimination in grapevine and crop performance. *Extended Abstracts 14th Meeting of ICVG, Locorotondo* 2003, 234-239.
- Martelli G.P., 1993. Rugose wood complex. In: Martelli G.P. (ed.). *Graft transmissible diseases of grapevine. Handbook for detection and diagnosis*, pp. 45-53. FAO Publication Division Rome, Italy.
- Martelli G.P., Jelkmann W., 1998. *Foveavirus*, a new plant virus genus. *Archives of Virology* 143: 1245-1249.
- Meng B., Johnson R., Peressini S., Forsline P.L., Gonsalves D., 1999a. Rupestris stem pitting associated virus-1 is consistently detected in grapevines that are infected with rupestris stem pitting. *European Journal of Plant Pathology* 105: 191-199.
- Meng B., Zhu H.-Y., Gonsalves D., 1999b. Rupestris stem pitting associated virus-1 consists of a family of sequence variants. *Archives of Virology* 144: 2071-2085.
- Meng B., Li C., Wang W., Goszczynski D., Gonsalves D., 2005. Complete genome sequences of two new variants of *Gra-*

- pevine rupestris stem pitting-associated virus* with comparative analyses. *Journal of General Virology* **86**: 1555-1560.
- Minafra A., Boscia D., 2003. An overview of Rugose wood-associated viruses: 2000-2003. *Extended Abstracts. 14th Meeting of ICVG, Locorotondo* 2003, 116-120.
- Murashige T., Skoog F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.
- Nitsch J.P., Nitsch C., 1969. Haploid plants from pollen grains. *Science* **163**: 85-87.
- Panattoni A., D'Anna F., Triolo E., 2006. Improvement in grapevine chemotherapy. *Extended Abstracts. 15th Meeting of ICVG, Stellenbosch* 2006, 139-141.
- Popescu C.F., Buciumeanu E.C., Visoiu E., 2003. Somatic embryogenesis, a reliable method for Grapevine fleck virus free grapevine regeneration. *Extended Abstracts 14th Meeting of ICVG, Locorotondo* 2003, 243.
- Rowhani A., Zhang Y.P., Chin J., Minafra A., Golino D.A., Uyemoto J.K., 2000. Grapevine rupestris stem pitting associated virus: population diversity, titer in the host, and possible transmission vector. *Extended Abstracts 13th Meeting of ICVG, Adelaide* 2000, 37.
- Wang Q., Mawassi M., Li P., Gafny R., Sela I., Tanne E., 2003. Elimination of grapevine virus A (GVA) by cryopreservation of in vitro-grown shoot tips of *Vitis vinifera* L. *Plant Science* **165**: 321-327.
- Zhang Y.-P., Uyemoto J.K., Golino D., Rowhani A., 1998. Nucleotide sequence and RT-PCR detection of a virus associated with grapevine rupestris stem-pitting disease. *Phytopathology* **88**: 1231-1237.

Received January 31, 2006

Accepted June 25, 2006