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Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine

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Abstract

The distribution of some grapevine viruses in flower explants, embryogenic and non-embryogenic calli, single somatic embryos and plants regenerated from embryogenic cultures was investigated by RT-PCR and ELISA. Immature anthers and ovaries of the cultivars Grignolino infected by GRSPaV, GLRaV-1 and GVA, Müller-Thurgau infected by GRSPaV and GLRaV-3 and Bosco infected by GRSPaV were cultivated on media inducing indirect somatic embryogenesis. Viruses were detected both in anthers and ovaries. Four months after culture initiation 65.6% of tested calli were infected by at least one virus; high percentages of virus infection were found in calli originating from ovaries. No virus was detected in calli tested 8 months after culture initiation, as well as in single somatic embryos or in embryo-derived plantlets. Somatic embryogenesis confirmed its effectiveness in eliminating phloem-limited grapevine viruses. Regeneration of RT-PCR negative plantlets occurred even when at least a sector of the callus was still infected: the mechanism whereby somatic embryos are freed of some viruses could be related to the rapid proliferation of embryogenic cells within the callus or to the origin of the embryogenic callus from virus-free cells within the original explant.

Introduction

Grapevines (*Vitis* spp.), along with other vegetatively propagated plants, are affected by many viral diseases. Grapevine leafroll (GLR) and Rugose wood complex are among the most harmful and widespread of graft-transmissible viral diseases. At least eight viruses (GLRaVs) have been associated with the GLR disease (Martelli et al., 2002). Rugose wood can be divided into four distinct disorders based on symptoms expressed on specific *Vitis* indicators: Kober stem grooving, LN 33 stem grooving, Corky bark and Rupestris stem pitting. *Grapevine virus A* (GVA) has been found closely associated with the Kober stem grooving disease (Chevalier et al., 1995), and according to several authors *Grapevine rupestris stem pitting-associated virus* (GRSPaV) is associated with the Rupestris stem pitting disease (Meng et al., 1999).

Establishment of vineyards free of GLRaVs, GVA and GRSPaV, as well as the other more dangerous grapevine viruses, is an important control measure. Several methods have been applied to eliminate viruses from infected grapevine clones, the most used being thermotherapy (Leonhardt et al., 1998) and meristem culture (Golino et al., 1998). Success varies with virus, grape cultivar and specific approach. Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programmes (Martinelli and Gribaudo, 2001), can efficiently

eliminate several grapevine viruses (Goussard et al., 1991; Schaefers et al., 1994). Similar results were obtained in Citrus (D'Onghia et al., 2001) and in sugarcane (Parmessur et al., 2002).

The mechanism whereby regenerated somatic embryos are freed of some viruses is not clear, though it has been noted that there was no translocation of phloem-limited viruses from infected tissue to somatic embryos (Goussard et al., 1991; Parmessur et al., 2002) while somatic embryogenesis alone was not effective in eliminating *Grapevine fanleaf virus* (GFLV) (Goussard and Wiid, 1992). D'Onghia et al. (2001) reported that all the embryogenic callus lines, derived from stigma and style cultures of *Citrus psorosis virus* (CPsV)-infected Citrus, were infected. Scagliusi et al. (2002) detected GLRaVs in non-embryogenic grapevine callus.

We have investigated the presence of GLRaV-1, GLRaV-3, GVA and GRSPaV at the various stages of grapevine somatic embryogenesis: flower explants (anthers and ovaries) which are the most valuable source for somatic embryogenesis in the genus *Vitis* (Martinelli and Gribaudo, 2001), embryogenic and non-embryogenic calli, single somatic embryos and plants regenerated from embryogenic cultures. We report the successful elimination of GLRaV-1, GLRaV-3, GVA and GRSPaV through somatic embryogenesis from Müller-Thurgau, Grignolino and Bosco, three grapevine wine cultivars.

Materials and methods

Plant material

Embryogenic cultures were initiated from immature flower explants of three virus-infected cultivars of *Vitis vinifera*: Grignolino infected by GRSPaV, GLRaV-1 and GVA, Müller-Thurgau infected by GRSPaV and GLRaV-3 and Bosco infected by GRSPaV. Their viral status was confirmed at the start of the work (see below). Inflorescences were collected in the vineyard 10–14 days before full bloom, when the pollen mother cells were in pre-meiotic phase (Gribaudo et al., 2004). Flower buds were chilled at 4 °C for 3 days and stamens (anthers plus filaments) and pistils (ovaries plus styles and stigmas) were excised. Below we refer to these simply as anthers and ovaries. The explants were cultivated on a Callus Induction (CI) medium (Franks et al., 1998; Gribaudo et al., 2004) containing Nitsch and Nitsch (1969) mineral salts, Murashige and Skoog (1962) vitamins, 6% sucrose and 0.3% gelrite, 4.5 μM 2,4-D and 8.9 μM BAP. Cultures were kept at 26 °C in the dark for 3 months. For maintenance of embryogenic cultures, subculturing was done monthly and an Embryo Differentiation (ED) medium (Franks et al., 1998; Gambino et al., 2005), containing the same basal medium supplemented with 10 μM NOA, 1 μM BAP, 20 μM IAA, 1% agar and 0.25% activated charcoal, was alternated every 2 months with the CI medium. Non-embryogenic calli were cultivated on CI medium with periodic subculture. About 5 months after culture initiation, single embryos were isolated from the embryogenic callus and transferred to the basal medium without Plant Growth Regulators (PGR), under light (photoperiod 16 h). Embryo-derived plantlets were micropropagated by culturing apical cuttings on a PGR-free, modified Murashige and Skoog (1962) medium with half-strength mineral salts, 2% sucrose, and 0.8% agar. Plants of Grignolino were acclimatized and transferred to the greenhouse.

Virus detection

The viral status of the mother plants was confirmed by ELISA for GFLV, GVA, GLRaV-1, GLRaV-3, *Grapevine fleck virus* (GFkV), and by RT-PCR for GRSPaV. Ovaries and anthers (sampled at culture initiation), embryogenic and non-embryogenic calli, and somatic embryos were tested by RT-PCR for presence of the viruses found in the mother plants. Additionally, single ovaries and anthers were screened for GVA, GLRaV-1 and GLRaV-3 infection by a tissue blot immunoassay (TBIA). The viral status of embryo-derived plantlets was assayed by both RT-PCR and ELISA, during *in vitro* culture and (for Grignolino) during later growth in the greenhouse.

ELISA

DAS-ELISA was used for GFLV, GLRaV-1 and GLRaV-3 tests, protein A DAS-ELISA for GVA and DASI-ELISA for GFkV. Polyclonal antisera and monoclonal antibodies were purchased from Agritest (Valenzano, Italy). Antigen samples were

obtained from woody material (scraping mature canes) of mother plants and of greenhouse-grown plants, and from whole micropropagated plantlets for plants grown *in vitro*. Tissues were macerated in extraction buffer (0.5 M Tris–HCl, 2% PVP-24, 1% PEG 6000, 0.14 M NaCl, 0.05% Tween 20, pH 8.2) at a dilution 1:10 (w/v). Optical densities were measured at 405 nm 3 h after addition of the substrate (*p*-nitro-phenylphosphate at 1 mg ml^{-1}). An ELISA sample was taken as positive if its OD_{405} value was at least three times the negative control value.

RT-PCR

Total RNA was extracted from mother plants (200 mg of phloem scraped from mature canes), and from samples collected at different culture stages. Anthers and ovaries were sampled (20 mg) at culture initiation. Whole embryogenic and non-embryogenic calli (10–50 mg), generated from single anthers or ovaries, were collected 4 and 8 months after culture initiation. Single embryos were sampled 1–2 months after transfer to the PGR-free medium. Regenerated plantlets were sampled (200 mg) during *in vitro* culture and during later greenhouse culture. All samples were immediately frozen and homogenised in liquid nitrogen. RNA was extracted following the protocol of MacKenzie et al. (1997) with modifications. Extraction buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5.0, 25 mM EDTA, 2.5% PVP-40, 2% sarkosyl and 1% 2-mercaptoethanol added just before use) was added to the homogenised sample and, after two extractions with chloroform/isoamyl alcohol (24:1 v/v), nucleic acids were precipitated with 0.7 volume of cold isopropanol. The pellet was resuspended in DEPC-water and 0.5 volume of 6 M LiCl added. The mixture was incubated overnight at 4 °C and RNA was selectively pelleted after centrifugation. The pellet was washed with ethanol, dried and resuspended in DEPC-water.

First-strand cDNA synthesis was performed using 500 ng of total RNA treated with DNase (Sigma-Aldrich, USA), 100 units of recombinant Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen Life Technologies, USA), 50 units of RNase inhibitor (RNase out, Invitrogen Life Technologies, USA), 0.5 mM of dNTPs, and 2.5 μM of random nonamers (Sigma-Aldrich, USA). The mix for reverse transcriptase (10 μl) was incubated for 50 min at 37 °C. A set of specific primers designed by Meng et al. (1999) was used for PCR amplification of GRSPaV. For diagnosis of GLRaV-1, GLRaV-3, GVA and for amplification of 18S rRNA, four specific primer pairs were designed after a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST), having about the same T_m values (63 °C) and being amplifiable at the same annealing temperature in PCR (Table 1).

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 $MgCl_2$ and 0.5 unit of Taq polymerase (PlatinumTaq polymerase, Invitrogen Life

Table 1. Primers for RT-PCR amplification of grapevine viruses and grapevine 18S rRNA

Target	Primers	Sequence[a](5′-3′)	Location[b]	Product size (bp)	Gene
GVA	GVA-H6481	AACCAACTGACGACGCTTCT	6481–6500	390	Coat protein
	GVA-C6870	ACGCGAAGTCGAACATAACC	6851–6870		
GLRaV-1	GR1-H7346	GCAACTGCAATTTCCACAGA	7346–7365	328	Coat protein
	GR1-C7673	CTTTCTCGTTCGGCTTCAAC	7654–7673		
GLRaV-3	GR3-H13384	ACGTTAAGGACGGGACACAG	13,384–13,403	367	Coat protein
	GR3-C13750	GCGCCCATAACCTTCTTAC	13,731–13,750		
GRSPaV[c]	RSP-H4373	GATGAGGTCCAGTTGTTTCC	4373–4392	339	Replicase
	RSP-C4711	ATCCAAAGGACCTTTTGACC	4692–4711		
rRNA 18S	18S-H325	AAACGGCTACCACATCCAAG	325–344	673	
	18S-C997	GCGGAGTCCTAAAAGCAACA	978–997		

[a]BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST) showed that all primers have 100% homology with their target sequence only.

[b]The reference accession numbers (NCBI) for determination of the primer position are: NC_003604 for GVA, AF195822 for GLRaV-1, NC_004667 for GLRaV-3, NC_001948 for GRSPaV and AF321266 for Vitis RNA 18S.

[c]Primers designed by Meng et al. (1999).

Technologies, USA). PCR was performed for 39 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min for GRSPaV, and for 35 cycles at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min for the other viruses. For each sample a control amplification of *Vitis* 18S rRNA was performed using specific primers in order to check for possible RT-PCR inhibitors or degradation of RNA. Reaction products were analysed in 1% agarose gels buffered in 0.5× TBE (1× TBE: 90 mM Tris–borate, 2 mM EDTA) and visualised by UV-light after staining with ethidium bromide.

TBIA

The tissue blot immunoassay was modified from the method of Martín et al. (2002). Single ovaries and anthers were gently pressed onto membranes of nitrocellulose of 0.45 μm pore size (Sigma-Aldrich, USA). The membranes were air-dried at room temperature and blocked for 30 min in TBS-milk buffer (TBS buffer [10 mM Tris, pH 7.4, 0.15 M NaCl] containing 5% non-fat dry milk). The membranes were then incubated for 3 h at room temperature in a 1/10,000 dilution of GVA, GLRaV-1 or GLRaV-3 antibodies conjugated with Alkaline Phosphatase (AP) (Agritest, Valenzano, Italy) in TBS-milk buffer. After three washes of 15 min each with TBS buffer plus 0.3% Tween 20, the membranes were equilibrated in substrate buffer (0.1 M Tris, pH 9.5) for 5 min before adding the substrate. AP activity was detected with the chromogenic substrate BCIP/NPT (Sigma-Aldrich, USA) according to the manufacturer's instructions. After rinsing in distilled water and drying on a paper towel the membranes were inspected for purple colour development.

Results

Somatic embryogenesis

Calli were obtained from both anther and ovary cultures with different efficiencies depending on genotype and explant type: on average about 25% anthers and 85% ovaries produced calli. Different types of callus were observed: a non-embryogenic dry and compact callus (Figure 1a), non-embryogenic watery and soft callus either yellow (Figure 1b) or brown (Figure 1c); a granular white

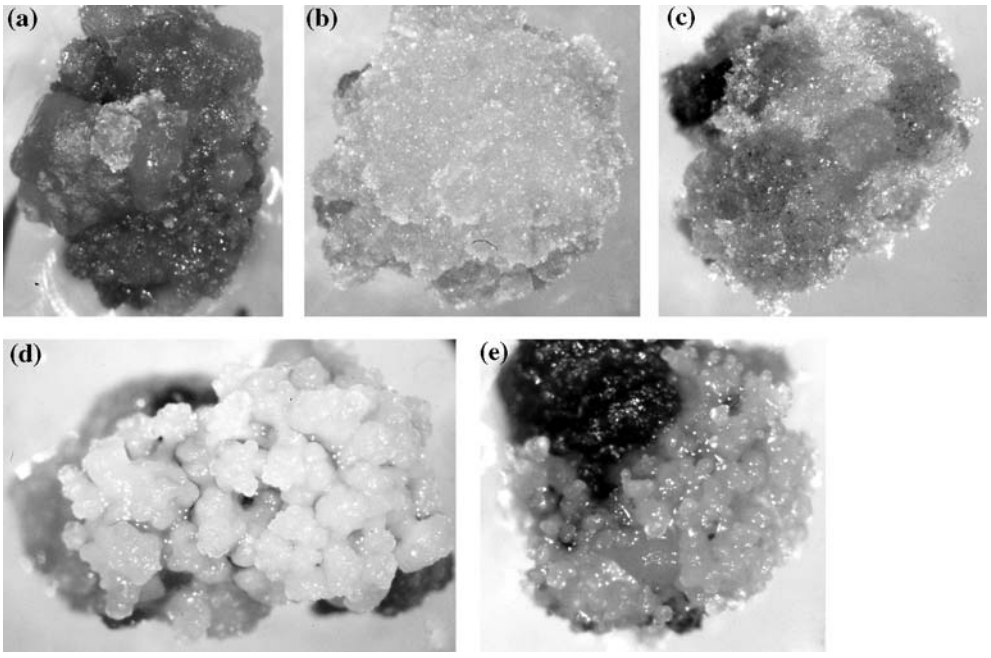

Figure 1. Phenotypes of non-embryogenic and embryogenic calli of Müller-Thurgau, Grignolino and Bosco, 3 months after culture initiation. Non-embryogenic dry and compact callus (a); non-embryogenic watery and soft callus, yellow (b) and brown (c); granular white embryogenic callus (d); embryogenic callus associated with dark callus (e).

or yellow pre-embryogenic callus (Figure 1d); an embryogenic callus associated with dark callus (Figure 1e). Within 3 months, 16.4%, 5.2% and 9.1% anthers of Müller-Thurgau, Grignolino and Bosco, respectively, originated embryogenic callus. Ovaries gave better results than anthers for Müller-Thurgau and Grignolino (40.6% and 7.5%, respectively) while in Bosco somatic embryos were produced from 7.4% ovaries. No morphological difference was noted between embryogenic cultures originated from ovaries or from anthers. Our protocol for long-term maintenance of embryogenic cultures allowed recovery of many embryos even from Grignolino. Non-embryogenic calli did not survive long on CI medium and after 6–7 months of culture all non-embryogenic calli had degenerated. Somatic embryo regeneration was asynchronous, and the first embryos were visible after about 3 months from culture initiation. The embryo morphology was heterogeneous and abnormal embryos were observed. Single embryos were isolated from embryogenic calli and transferred to the PGR-free medium: shoots emerged after 1–2 months of culture under the light regime described above.

Virus detection

Although in the laboratory RNA is routinely extracted from 200 mg of tissue, during the present research the available amount of tissue was often considerably lower: 20 mg for flower explants (anthers and ovaries), up to 50 mg for calli. The corresponding PCR bands in agarose gel had satisfactory intensities and the designed primer pairs amplified fragments with the expected sizes under the RT-PCR conditions used. The sensitivity of the assays used was previously ascertained by serial dilutions of RNA extracted from infected plants: RT-PCR detected presence of single viruses at 10^{-5} dilution. ELISAs and RT-PCRs on mature canes confirmed the virus infection of the field plants used as source material. All the viruses detected in the canes of mother plants were also found by RT-PCR in their anthers and ovaries at culture initiation (Figure 2); virus presence in single ovaries and anthers was confirmed by TBIA performed on 50 samples of Grignolino and Müller-Thurgau.

Different types of callus developed from anthers or ovaries were tested by RT-PCR 4 and 8 months after culture initiation; results are shown in Table 2. Four months after culture initiation, high percentages of GLRaV-3, GVA and GRSPaV infection were found in calli from ovary cultures while in anther-derived calli a more limited presence of viruses was observed. GRSPaV often showed higher levels of infection compared with the other viruses. Distribution of the viruses in tissues seemed to be independent of callus morphology (data not shown). No virus was detected in embryogenic calli tested again 8 months after culture initiation; at this time all non-embryogenic calli had degenerated.

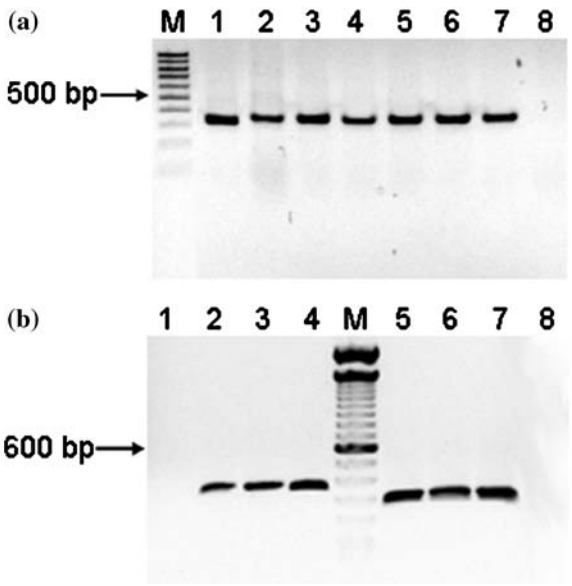

Figure 2. Agarose gel analysis of RT-PCR assays with primers specific for GRSPaV (a), GVA and GLRaV-1 (b), performed on anthers and ovaries (images in negative contrast). (a) Lane 1, GRSPaV positive sample; lanes 2–3, anthers and ovaries, respectively, from Grignolino; lanes 4–5, anthers and ovaries from Müller-Thurgau; lanes 6–7, anthers and ovaries from Bosco; lane 8, negative control (GRSPaV negative sample); M, molecular weight markers. (b) Lanes 1–4 analyses of RT-PCR assays with GVA specific primers; lane 1, negative control (GVA negative sample); lanes 2–3, anthers and ovaries from Grignolino; lane 4, GVA positive sample; lanes 5–8 analyses of RT-PCR assays with GLRaV-1 specific primers; lane 5, GLRaV-1 positive sample; lanes 6–7, anthers and ovaries from Grignolino; lane 8, negative control (GLRaV-1 negative sample); M, molecular weight markers.

The somatic embryos analysed by RT-PCR were randomly selected among various developmental stages observed (heart-shaped, torpedo and cotyledonary). All embryos tested were virus-negative (Table 2 and Figure 3). We found

Table 2. Results of RT-PCR on calli, single somatic embryos and embryo-derived plants

Explants	Months from culture initiation	Number of infected samples/number of samples tested			
		GLRaV-1 (Grignolino)	GLRaV-3 (Müller-Thurgau)	GVA (Grignolino)	GRSPaV (Grignolino, Bosco and Müller-Thurgau)
Non-embryogenic calli from anthers	4	0/12	0/6	2/12	14/32
Non-embryogenic calli from ovaries	4	6/11	8/10	9/11	28/35
Embryogenic calli from anthers	4	0/5	1/5	1/5	8/15
Embryogenic calli from ovaries	4	4/4	3/5	1/4	8/14
Embryogenic calli from anthers and ovaries	8	0/10	0/10	0/10	0/30
Somatic embryos	5–7	0/32	0/10	0/32	0/74
In vitro plantlets	12	0/46	0/49	0/46	0/95
Greenhouse plants	24	0/16	–	0/16	0/16

Cultures originated from anthers and ovaries of Müller-Thurgau (originally infected by GLRaV-3 and GRSPaV), Grignolino (infected by GLRaV-1, GVA and GRSPaV) and Bosco (infected by GRSPaV).

no correlation between virus infection and embryo morphology (i.e., normal and abnormal somatic embryos). Plantlets deriving from somatic embryos were tested three times by ELISA and once by RT-PCR: the results were always negative. Results obtained for *in vitro*-cultured plantlets were confirmed by ELISA and RT-PCR 2 years later on embryo-derived, greenhouse-grown Grignolino plants (Table 2).

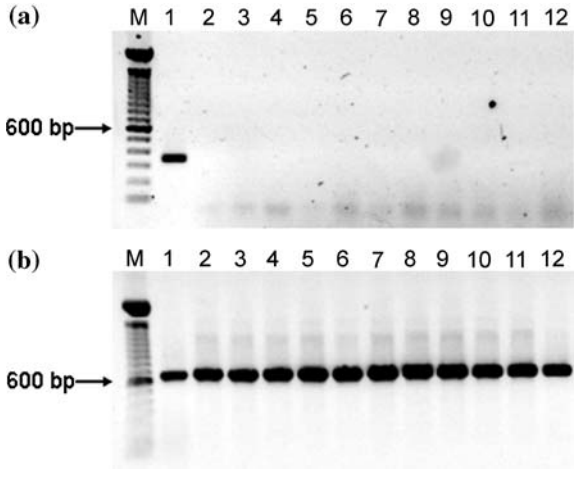

Figure 3. Agarose gel analysis of RT-PCR assays with primers specific for GLRaV-3 (a) and 18S rRNA (b), performed on single somatic embryos (images in negative contrast). (a) Lane 1, GLRaV-3 positive sample; lanes 2–11 embryos of Müller-Thurgau; lane 12 negative control (GLRaV-3 negative sample); M, molecular weight markers. (b) The same samples analysed in (a) amplified with specific primers for 18S rRNA.

Discussion

In vitro-grown plants can have higher virus titre than similar greenhouse plants, and micropropagated infected explants of grapevine have been proposed as a source for virus purification (Monette and James, 1990). On the contrary concentration and persistence of viruses in *in vitro*-cultured calli are controversial topics. In general, it is well known that virus levels in cultured cells can be very low and sometimes the virus may be lost (see the review of Wang and Hu, 1980). Salati et al. (1993) detected closteroviruses in callus cultures from only two of five infected grapevines. Scagliusi et al. (2002) found that GLRaV-3 concentration in callus from infected grapevine plants was high enough over many successive generations to propose the use of this callus for studying the virus.

In our study, the results of assays for GLRaV-1, GLRaV-3, GVA and GRSPaV indicated that all the original explants (anthers and ovaries) were infected. RT-PCR results were confirmed by TBIA, a method used for rapid detection of several viruses (Lin et al., 1990; Martin et al., 2002). The percentages of infected calli varied according to

their origin. Ovaries are larger than anthers and this may entail a higher initial viral inoculum, with consequently more frequent virus detection in ovary-derived calli after 4 months of culture. Nevertheless, after 8 months of culture no virus was detected in embryogenic ovary- or anther-derived calli.

All sampled embryos were virus-free, while 4 months after culture initiation at least one virus was detected in more than 65% of embryogenic calli. Therefore regeneration of healthy plantlets could begin even when at least a sector of the callus was still infected. Several workers have noted that calli originating from virus-infected plants are a mosaic of infected and uninfected cells (Wang and Hu, 1980; Walkey, 1991). Also in grapevine callus the distribution of GLRaV-3 particles appeared to be uneven, with high concentrations of virus in some groups of cells (Scagliusi et al., 2002). According to George and Sherrington (1984), the meristematic nature of callus tissue can inhibit virus replication, especially in cytokinin-containing media.

However, the regeneration of healthy embryos is related to virus type and involves mechanisms of virus movement in the tissues. As a rule, phloem-limited viruses are not seed-transmissible as they cannot enter zygotic embryos (which lack vascular contact with the mother plant) as in the case with Citrus nucellar embryos (Bos, 1999). Seed transmission via the embryo and via nucellar tissues seems to require infection of meristematic tissue prior to differentiation into ovule and cytological seclusion of the embryo from the mother plant (Bos, 1999). No vascular connection between neighbouring embryoids or between grapevine somatic embryos and the parent tissue was observed by Newton and Goussard (1990). According to Goussard et al. (1991) no translocation of GLRaVs occurred from infected tissues via proliferating callus (without vascular tissue) to somatic embryoids.

Restriction of some viruses to certain tissues (e.g. phloem-limited viruses) does not necessarily exclude other translocation pathways such as through plasmodesmata, but this is a slow movement (Mitchell et al., 1960). Cytoplasmic connections between cells are known to occur in callus culture (Spencer and Kimmins, 1969; Brighigna et al., 1992). The multiplication rate of *Tobacco mosaic virus* (TMV) in tobacco calli and the velocity of its translocation varied depending on the types of callus (Omura and Wakimoto, 1978). These authors assumed that in compact callus rapid virus movement occurred through tracheid-like cells and/or sieve elements and slow movement through plasmodesmata. In our cultures embryogenic and non-embryogenic calli could originate from virus-infected anther and ovary cells but virus presence could be limited to the original explant tissues and possibly to the old cells of the callus, invaded through plasmodesmata. The rapidly proliferating young cells and somatic embryos regenerated from these may escape virus invasion.

Alternatively, embryogenic callus may originate from virus-free cells within the original explants. Newton and Goussard (1990) observed that embryogenic cells arose from specific parts of the anthers and filaments. Embryogenic callus from ovule cultures originated from the nucellar tissue, which lacks vascular connection to the mother plant and is likely to be virus-free (Schaefers et al., 1994).

An ultrastructural and cytopathological study of infected calli can provide clear answers. However, the above hypotheses cannot fit all types of virus. For example, GFLV particles, which are not restricted to vascular tissue, readily invade plant meristems and are highly seed-transmissible: GFLV was eliminated by somatic embryogenesis only if this was combined with thermotherapy (Goussard and Wiid, 1992).

The present work confirms the effectiveness of somatic embryogenesis in eliminating GLRaV-1, GLRaV-3, GVA and GRSPaV. The regeneration protocol adopted here has proved useful for several *V. vinifera* cultivars (Gribaudo et al., 2004). However, the possibility of somaclonal variation and/or juvenility characters needs to be carefully ascertained in regenerated plantlets; with this aim, an experimental vineyard of Grignolino from somatic embryos was planted in spring 2005. In this vineyard the eventual re-infection of regenerated plants will also be monitored.

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