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Elimination of *Grapevine fanleaf virus* from three *Vitis vinifera* cultivars by somatic embryogenesis

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Abstract Indirect somatic embryogenesis was tested as a method for eradication of *Grapevine fanleaf virus* (GFLV) in three grapevine cultivars. Reverse transcriptase-polymerase chain reaction for GFLV detection was performed on tissues sampled at various steps of the embryogenic process: flower explants, embryogenic and non-embryogenic calli, single somatic embryos and regenerated plants. The virus was detected in all tested anthers and ovaries, while only one sample out of 63 regenerated plantlets was positive in the assay. Although GFLV was able to invade embryogenic calli and embryo-derived plantlets, in our experimental conditions GFLV eradication was obtained by somatic embryogenesis alone, without using heat therapy, with a success close to 100%. Assessment of the phytosanitary status of regenerated plants confirmed that GFLV was not detectable in the newly formed leaves 2 years after their transfer to greenhouse conditions.

Keywords GFLV · Grapevine · RT-PCR · Somatic embryos · Virus eradication

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Grapevine fanleaf virus (GFLV), a soil-borne nepovirus, is the main causal agent of grapevine fanleaf disease, one of the most damaging and widespread viral diseases affecting grapevine. In sensitive cultivars this nepovirus can cause rapid death of young plants or a progressive decline over several years (Martelli 1993). GFLV is spread both via propagating material and nematode vectors belonging to the genus *Xiphinema* (particularly *X. index*). Establishment of vineyards free of GFLV and other detrimental viruses, such as *Grapevine leafroll associated virus-1, -2* and *-3, Arabis mosaic virus*, *Grapevine virus A*, is an important control measure. Several methods have been used to eliminate viruses from infected grapevine clones. Meristem tip culture is particularly effective in eliminating phloem-limited viruses, while thermotherapy is normally required for the elimination of other viruses such as nepoviruses, that readily invade plant meristems. Somatic embryogenesis, usually adopted to regenerate plantlets in biotechnological breeding programmes, was used to eradicate viruses from citrus (D'Onghia et al. 2001) and sugarcane (Parmessur et al. 2002). In grapevine it efficiently eliminated several phloem-limited viruses (Goussard et al. 1991; Gambino et al. 2006; Gribaudo et al. 2006). An attempt to eradicate GFLV through somatic embryogenesis was reported by Goussard and Wiid (1992) who eliminated the virus only if somatic embryogenesis was combined with thermotherapy of the explants.

In the present work we investigated the presence of GFLV at various stages of grapevine somatic embryogenesis: flower explants (anthers and ovaries), embryogenic and non-embryogenic calli, somatic embryos and embryo-derived plantlets.

Embryogenic cultures were initiated from flower explants of three Italian cultivars of *Vitis vinifera*: Cari, Provinè and Roussan. The mother plants were infected by GFLV, as confirmed by serological assays. Immature anthers and ovaries were isolated from inflorescences collected in vineyards and indirect somatic embryogenesis was induced as previously described (Gribaudo et al. 2004). Floral explants were cultivated on a callus induction medium containing 4.5 μM 2,4-D and 8.9 μM BAP. After 3 months, calli were transferred to an embryo differentiation medium containing 10 μM NOA, 1 μM BAP, 20 μM filter-sterilised IAA, and 0.25% activated charcoal, for 2 months. Cultures were kept at 26°C in the dark. Plant development was obtained adopting the protocol of Franks et al. (1998) with modifications (Gambino et al. 2005). Embryo-derived plantlets were micropropagated by culturing apical cuttings on a plant growth regulator (PGR)-free medium, thus giving rise to individual lines. In spring the plants were acclimatised and transferred to a cool greenhouse. The greenhouse received minimum heating during winter so the lignified plants could enter dormancy.

The viral status of the mother plants was confirmed by ELISA (Gambino et al. 2006). Anthers and ovaries, embryogenic and non-embryogenic calli, somatic embryos and plants were tested by reverse transcriptase-polymerase chain reaction (RT-PCR) for presence of GFLV. Anthers and ovaries were sampled (20 mg) at culture initiation. Whole embryogenic and non-embryogenic calli (10 to 50 mg), generated from single anthers or ovaries, were collected 4 months after culture initiation. Single somatic embryos were sampled 6 to 8 months after culture initiation. Regenerated plantlets were sampled (200 mg) during micropropagation. In addition, embryo-derived plants growing in the cool greenhouse were tested. Newly developed leaves (200 mg) were collected from 10, 9 and 7 plants respectively of Cari, Proviné and Roussan at bud break after one dormancy period, and 10 plants of Cari after 2 years.

Total RNA from anthers and ovaries was extracted as previously described (Gambino et al. 2006). RNA from other samples was extracted following the protocol of Chang et al. (1993). First-strand cDNA synthesis was performed as previously described (Gambino et al. 2006). A set of specific primers (Forward-PM1 5′- CCCTCAACCAGAGCCAATTA-3′ and Reverse-PM2 5′- GGCATTGGTAGAGGCAA CAT-3′) was designed on conserved regions of the movement protein gene of GFLV using the software Primer 3 (Rozen and Skaletsky 2000) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The two primers allow for the amplification of a 378 bp fragment. Additionally a second primer set (Forward 5′- ATGCTGGATATCGTGACCCTGT -3′ and Reverse 5′- GAAGGTATGCCTGCTTCAGTGG -3′), designed on conserved regions of the GFLV RNA-dependent RNA polymerase gene to generate a 118 bp fragment (Gambino and Gribaudo 2006), was also used for PCR amplification of cDNA from regenerated plantlets. 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 Technologies, Carlsbad, CA). PCR was performed for 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. For each sample a control amplification of *Vitis* 18S rRNA was performed using specific primers (Gambino and Gribaudo 2006), in order to check for possible RT-PCR inhibitors or degradation of RNA. Reaction products were analysed in 2% agarose gels buffered in TBE (45 mM Tris-borate, 1 mM EDTA) and visualised by UV-light after staining with ethidium bromide. The sensitivity of the RT-PCR assays was previously ascertained (Gambino and Gribaudo 2006) by serial dilutions of RNA extracted from infected plants: RT-PCR detected presence of GFLV at 10^{-5} dilution.

Calli were obtained from both anther and ovary cultures with different efficiencies depending on genotype and explant type (Table 1). The adopted protocol for long-term maintenance of embryogenic cultures allowed the recovery of several embryos even from cultures of Cari and Roussan (anthers), which had a relatively low embryogenesis efficiency from primary explants.

ELISA and RT-PCR on mature canes confirmed the GFLV infection of the field plants used as source material and the virus was also found by RT-PCR in their anthers and ovaries at culture initiation. Due to the low embryogenesis efficiency of Cari and Roussan,

Table 1 Frequency (%) of somatic embryogenesis in ovary and anther-derived calli of *V. vinifera* cvs Provinè, Cari and Roussan after 3 months of culture

Cultivar	Explant type	Embryogenesis (%)
Provinè	Anther	9.1
	Ovary	13
Cari	Anther	0.4
	Ovary	1.4
Roussan	Anther	0.8
	Ovary	9.1

For each cultivar at least 550 anthers and 100 ovaries were cultivated.

calli and somatic embryos of Provinè only were analysed by RT-PCR. Four months after culture initiation, high percentages of GFLV infection were found in calli from anther and ovary cultures (Table 2). These results are similar to those obtained previously (Gambino et al. 2006) for the phloem-limited viruses GLRaV-1, GLRaV-3, GVA and GRSPaV. In that case the percentage of infected calli varied according to their origin and it was hypothesised that the larger size of ovules, compared to anthers, may have entailed a higher initial viral inoculum with consequently more frequent virus detection in ovary-derived calli. GFLV was detected in some somatic embryos of Provinè (Table 2) and in one sample out of 63 plantlets regenerated from somatic embryos of Provinè, Cari and Roussan (Table 3). No discrepancy was observed when a second primer pair for GFLV detection by RT-PCR assay was used. Additional RT-PCR analyses were performed on embryo-derived plants cultivated in the greenhouse after one (Cari,

Table 2 Results of RT-PCR on calli and somatic embryos originated from anthers and ovaries of Provinè originally infected by GFLV

Explants	Number of infected samples/ number of samples tested
Non-embryogenic calli from anthers	6/11
Non-embryogenic calli from ovaries	10/10
Embryogenic calli from anthers	2/6
Embryogenic calli from ovaries	2/2
Somatic embryos	2/9

Table 3 Elimination of GFLV: cultivars tested, total number of lines obtained and number of lines found to be GFLV-free in RT-PCR assays

Cultivar	N° lines obtained	N° lines cleaned
Provinè	34	33
Cari	16	16
Roussan	13	13

Proviné and Roussan) or two (Cari) dormancy periods; GFLV was never detected in any of the samples, thus confirming the results of the initial assays. An example of agarose gel analysis of RT-PCR assays with primers specific for GFLV (and for *Vitis* 18S rRNA as a control for RNA amplification) is shown in Fig. 1.

The average percentage of infected plants regenerated from GFLV-infected explants was 1.6%. These data differ from those of Goussard and Wiid (1992) who did not obtain any sanitated plant unless the anther cultures were previously heat-treated. The reason for this diversity is questionable. Both studies show that the GFLV is able to invade embryogenic calli and regenerated plantlets. However there are important differences between the two protocols, particularly in the composition of media used to induce indirect somatic embryogenesis from floral explants. In our protocol PGRs were used at high concentrations for prolonged periods of time: BAP in the callus induction medium, and auxins (NOA and

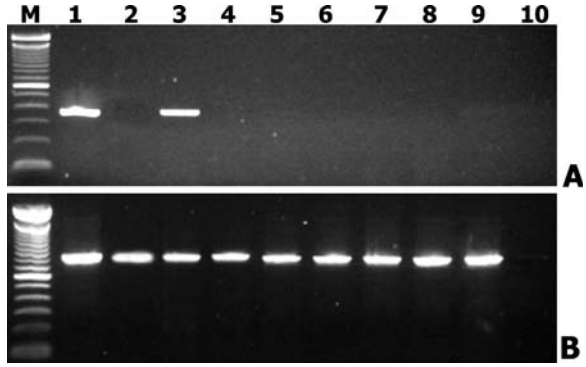

Fig. 1 Agarose gel analysis of RT-PCR assays with primers specific for GFLV (**A**) and 18S rRNA (**B**), performed on embryo-derived plantlets. **A** *lane 1*, GFLV positive sample; *lanes 2* to *5*, plantlets of Provinè; *lanes 6* to *9*, plantlets of Cari; *lane 10*, water; *M*, 100 bp DNA ladder. **B** the same samples analysed in panel **A** amplified with specific primers for 18S rRNA

IAA) in the embryo differentiation medium. There are indications that treatments with cytokinins have detrimental effects on some viruses (Johnstone and Wade 1974; Clarke et al. 1998), as well as high concentrations of IAA (Clarke et al. 1998). Unfortunately the effects of exogenous cytokinins and auxins on virus replication remain to be fully elucidated (see the review of Jameson and Clarke 2002). In addition to direct and/or indirect effects of PGRs on viruses present in the cultured tissues, the lack of vascular connection between grapevine somatic embryos and the parent tissue (Newton and Goussard 1990) can be considered a reason for the sanitation occurring during cultures. However this factor seems to be more important for phloem-limited viruses than for GFLV, whose particles are not restricted to vascular tissue and readily invade plant meristems. An ultrastructural and cytopathological study of infected calli may provide clear answers.

This work presents for the first time the elimination of GFLV through somatic embryogenesis alone from three grapevine cultivars with a success percentage close to 100%. Somatic embryogenesis is more technically difficult compared to other sanitation techniques and it is largely genotype-dependent, although the regeneration protocol reported here proved suitable for several grapevine cultivars (Gribaudo et al. 2004). Further studies on different infected genotypes may confirm the efficacy of this protocol in GFLV eradication. Since somatic embryogenesis has already proven to be very effective in the elimination of the most important phloem-limited grapevine viruses, and considering the large number of viruses or virus-like agents that infect grapevine, it will be interesting to investigate this technique also for the eventual concurrent eradication of minor viral diseases.

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