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RESEARCH ARTICLE

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Mature seeds for *in vitro* sanitation of the *Grapevine leafroll associated virus* (GLRaV-1 and GLRaV-3) from grape (*Vitis vinifera* L.)

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Abstract

The conservation of old grapevine varieties is important since they are adapted to specific climate conditions and may carry genes interesting to breeders. As virus infection is common in grapevine varieties, the use of virus free materials is of great importance. In this work, we used somatic embryogenesis for the sanitation of GLRaV-1 and GLRaV-3 viruses that were found after analyzing the putative presence of the five most common, economically important grape viruses by real-time multiplex RT-PCR in the old cultivar "Grumet Negre". Unopened and opened inflorescences, fecundated ovaries, and, also, mature seeds were used as starting explants. Explants were cultured on plates with two embryogenesis induction media (Nitsch & McCown Woody plant medium) that contained the growth regulator thidiazuron and differed in their salt and vitamin compositions. One half of each kind of explant was cut prior to being cultured. After five months of culture, embryos had only developed from seeds that were cut previous to sowing. To the best of our knowledge, this is the first time that mature seeds have been used for inducing embryogenesis in grape. A total of 42% of the embryos transferred to tubes for germination regenerated into normal plantlets. The absence of both the GLRaV-1 and GLRaV-3 viruses in all regenerated plants was confirmed by real-time uniplex RT-PCR. So, this protocol can be used for sanitation and also for micropropagation.

Additional key words: embryogenesis; cultivar Grumet Negre; mature seeds; virus-free; micropropagation.

Abbreviations used: ArMV (*Arabis mosaic virus*); EIM (embryogenesis induction medium); GA_3 (gibberellic acid); GFkV (*Grapevine fleck virus*); GFLV (*Grapevine fanleaf virus*); GLRaV-1 (*Grapevine leafroll associated virus-1*); GLRaV-3 (*Grapevine leafroll associated virus-3*); GN (Grumet Negre); RT-PCR (reverse transcription-polymerase chain reaction); TDZ (thidiazuron)

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Introduction

Vitis vinifera L. clones selected for their high yield and quality are used in viticulture worldwide. However, sustaining native biodiversity and recuperating autochthonous cultivars that may carry interesting genes and/or are adapted to specific conditions is reported as a necessity by ecologists, naturalists, and breeders (Dickinson, 2013; Karvonen, 2014).

In general, autochthonous grapevine cultivars present a high incidence of virus infection (Laimer *et al.*, 2009; Bertolini *et al.*, 2010). In many cases, plants of old grapevine varieties, even plants that do not manifest symptoms, are virus-infected (Borroto-Fernandez *et al.*, 2009). Thus, in order to initiate the development and conservation of grapevine germplasm it is necessary to check the putative presence of the viruses and to achieve virus-free plants. These plants can be micropropagated, conserved in *in vitro* germplasm banks, and transferred to the field for cultivation. Virus sanitation is very important because the long-distance spread of grape-

vine viruses occurs primarily by the propagation of infected plant material. Thus, once a viral disease is established in the field, there is no treatment or cure. The EU Directive 2002/11/EC rules require that the initial plant material for vegetative propagation is free of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus-1* (GLRaV-1), and *Grapevine leafroll associated virus-3* (GLRaV-3).

Different *in vitro* culture techniques can be applied in order to sanitize virus infected material. The most commonly used procedures for viruses and viroids are meristem culture, somatic embryogenesis combined or not with thermotherapy, and chemotherapy (Panattoni *et al.*, 2013; Parštein *et al.*, 2013; Cheong *et al.*, 2014). For instance, in grape, meristem culture has been used for the production of viroid-free grapevines (Duran-Vila *et al.*, 1988) and also to clean plants infected with GFLV, GLRaV-1, and GLRaV-3 (Youssef *et al.*, 2009; Shatnawi *et al.*, 2011). In order to cure plants of GFLV, embryogenesis was used alone (Gambino *et al.*, 2009) or in combination with thermotherapy (Goussard & Wiid, 1992). Also, this technique was used to produce vines free of GLRaV (Goussard *et al.*, 1991; Gambino *et al.*, 2006), GFkV (Popescu *et al.*, 2003) or ArMV (Borroto-Fernandez *et al.*, 2009). Recently, elimination of the *Grapevine rupestris stem pitting-associated virus* (GRSPaV) by *in vitro* chemotherapy was reported by Skiada *et al.* (2013).

The aim of this work was to develop an embryogenesis protocol, using common explants and also mature seeds, for sanitation of the old grapevine cultivar 'Grumet Negre' (GN), a minor variety used as a table grape and for fermentation in the past, cultivated in the province of Alicante and other Mediterranean areas (García-Gracián, 1992; Bosch-Rododera, 1999; Favà-Agud, 2001).

Material and methods

Plant material

The old cultivar 'Grumet Negre' (GN), produces lilaceous grapes and large clusters, generally not very compact and small, compared to other varieties of table grapes. In the International list of vine varieties and their synonyms (www.oiv.int), the cultivar 'Grumet Negre' has the putative synonyms 'Valencí RS' or 'Valanci Negro' in Argentina and 'Valensi Noir' in France and Morocco. Explants of GN were collected from a vine in Penàguila (Alicante, Spain) where it is known as 'Valencí Negre'.

Analysis of mother plants

Leaves collected from a plant of cultivar GN were used. Extracts were prepared from leaves by grinding (at approx. 1/20, w/v) in PBS buffer, pH 7.2, supplemented with 0.2% (w/v) DIECA and 2% (w/v) polyvinyl-pyrrolidone (PVP-10), in individual plastic bags with a net (Plant Print Diagnostics) to avoid contamination. The total RNA was extracted from 200 µL of crude extract using the Ultraclean Plant RNA isolation kit (MoBio). The real-time multiplex RT-PCR was performed according to López-Fabuel *et al.* (2013) for the simultaneous detection of ArMV, GFLV, GFkV, GLRaV-1, and GLRaV-3. Amplifications were performed in a StepOne Plus thermocycler (Applied Biosystems). The real-time multiplex RT-PCR reaction mixture consisted of a 25-µL final volume, containing 1x AgPath-ID One-step RT-PCR buffer (Ambion) and 1.5x AgPath-ID One-step RT-PCR enzyme mix (Ambion); 5 µL of sample; 400 nM of GFLV, ArMV, GFkV, and GLRaV-1 primers; 800 nM of GLRaV-3 primers; and 200 nM of each probe. The amplification protocol consisted of an RT step at 45°C for 10 min and a denaturation step at 95 °C for 10 min, followed by 45 cycles of amplification (95 °C, 15 s; 50 °C, 15 s; and 60°C, 60 s). Data acquisition and analysis were performed using the StepOne Plus 2.0 software. The default threshold set by the machine was slightly adjusted above the noise to the linear part of the growth curve, at its narrowest point according to the StepOne Plus manufacturers. Positive viral isolates maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA) were included as a positive control. Positive explants from cultivar GN were confirmed by real-time uniplex RT-PCR using the primers which amplified GLRaV-1 and GLRaV-3 sequences. The real-time uniplex RT-PCR conditions were the same as those of the multiplex, in a total volume of 12 µL including 3 µL of sample.

Embryogenesis protocol for sanitation of grape

Embryogenesis induction and plant conversion. Different types of explants were used to induce embryogenesis in GN: unopened and opened grapevine inflorescences, fecundated ovaries, and seeds. The length × width was 2.0 × 1.5 mm for unopened inflorescences; opened inflorescences were slightly smaller, and fecundated ovaries were 3 mm long × 3 mm wide. Seeds were obtained from grapes with a mean weight of 0.70-0.85 g and were 6.0-7.0 mm long × 3.0-3.5 mm wide. The seeds had been extracted by hand and were sterilized like the rest of the explants, by submersion in 0.4% sodium hypochlorite (40 g/L of active chlorine)

for 10 min, before being rinsed three times in sterile distilled water. Half of the explants of each type were cut transversally previous to culture on media for embryogenesis induction. Two different media, EIM1 and EIM2, were used: EIM1 uses the salts and vitamins of Nitsch medium (Nitsch & Nitsch, 1969) whereas EIM2 uses the salts and vitamins of the McCown Woody plant medium (Lloyd & McCown, 1980). Both media contained: 4% sucrose, 0.01% PVP-10, 0.75% plant agar, 0.2% activated charcoal, and 0.9 µM TDZ (sterilized by filtration and added to the sterile medium).

After three months of culture on EIM1 or EIM2 medium, explants were transferred to fresh media without growth regulators. After five months of initial culture, we noted the number of explants that were: 1) dead, 2) green without apparent growth (non-responding), or 3) callus. A total of 130 plates (with 10 explants/plate) were randomly distributed, to evaluate the percentages of dead explants, non-responding explants, and explants with callus. At least six plates per combination of effects were included. The percentage of explants with embryos and the number of embryos per responding explant were also calculated.

The pH of all the media was adjusted to 5.8 before sterilization at 121 °C for 20 min, and the cultures were incubated in a growth chamber at 26 ± 2 °C under dark conditions.

Fifty embryos at different development stages, induced in either EIM1 or EIM2, were transferred to tubes with 16 mL of MW medium, for plant conversion. This medium consisted of McCown Woody plant salts and vitamins, 2% sucrose, 0.01% PVP-10, and 0.75% plant agar, supplemented with 1.4 µM GA_3. During the first week, the tubes were incubated under low-light conditions and then transferred to standard conditions: 16-h photoperiod with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 µmol/m^2/s).

Statistical analysis. The percentages of dead explants, non-responding explants, and explants with callus were analyzed using the following model:

$$y_{ijkl} = \mu + O_i + M_j + C_k + e_{ijkl}$$

where µ is the general mean, O_i is the ovary stage effect (unopened inflorescences, opened inflorescences, fecundated ovaries, and seeds), M_j is the culture media effect (EIM1 with Nitch salts and vitamins and EIM2 with Woody salts and vitamins), C_k is the effect of cutting (yes or no), and e_{ijkl} is the random residual term.

Embryos were only obtained from seeds that had been cut transversally. So, the model used to analyze the percentage of total embryos, the direct embryos and the total number of embryos per callus just included the effect of the culture media.

The SAS statistical package (SAS Inst. Inc., Cary, NC, USA) was used for these analyses.

Virus analysis of regenerated plants. Analysis of regenerated plants was carried out by performing uniplex real-time RT-PCR for the GLRaV-1 and GLRaV-3 viruses, as described for the mother plants. Fifteen plants were analyzed when they had developed 4-5 leaves. These plants were maintained *in vitro* and were transferred to fresh medium when they reached about 12 cm (approx. every 2 months). A sample of plants was acclimatized and cultured in a greenhouse and analyzed again in order to test the putative presence of virus after 5 months of culture.

Results

Virus analysis in mother plants

The real-time multiplex RT-PCR amplified the GLRaV-1 and GLRaV-3 viruses in cultivar GN. The real-time uniplex RT-PCR confirmed the presence of both viruses, as well as the absence of the GFLV, GFkV, and ArMV viruses.

Embryo induction

After 2 months of culture, differences in appearance were observed among the cultured explants: browning of inflorescences, green inflorescences, and callus. With respect to the fecundated ovaries, some showed no growth and others were thickened. At this time, the first embryos (which had germinated precociously) were observed, for three seeds that had been cut prior to sowing.

At 5 months, the percentages of explants that were dead, did not grow, or presented callus formation were noted (Table 1). There were different percentages of dead explants depending on the stage of ovary development. A high percentage of dead explants (around 50%) was observed in unopened inflorescences, whereas around 10% of explants were dead in opened inflorescences and less than 5% in fecundated ovaries and seeds. However, the percentage of dead explants was not affected by the growth medium or by cutting. The percentage of non-responding explants was also affected by the stage of ovary development; the lowest percentage, around 15%, was observed for the earliest embryonic stage of the ovary (unopened inflorescences), while higher values were obtained for the other stages of the ovary. Moreover, the EIM1 medium gave a lower percentage of non-responding explants than EIM2 (16.5% *vs.* 38.3%); therefore, EIM1 medium was superior. Analogous to this, the mean percentage of

non-responding explants was lower after cutting, with respect to the uncut explants (17.1% *vs.* 37.8%, respectively), indicating a positive effect of cutting. Callus formation was greater in opened inflorescences and fecundated ovaries than in unopened inflorescences and seeds. Moreover, explants cultured on EIM1 medium showed a higher percentage of callus (61.9%) compared to those cultured on EIM2 medium (40.1%). However, no cutting effect on the percentage of callus was obtained. At this time, many embryos obtained from cut seeds were growing in the tubes.

The percentage of total embryos was higher when the EIM1 medium was used, compared to EIM2 (64.6% *vs.* 48.5%, respectively; Table 2). Direct embryogenesis (from explants without calli), measured as the percentage of direct embryos with respect to the total number of embryos, was higher in EIM2 than in EIM1 (86% *vs.* 75%, respectively). Taking into account the result obtained in the present work, EIM2 increased the direct embryogenesis compared to EIM1. So, the EIM2 medium should be used in order to obtain higher direct embryogenesis for cultivar GN. The difference between direct and indirect regeneration is shown in Fig. 1 (A-C). There was no difference in the total number of embryos per callus when the culture media EIM1 and EIM2 were compared (4.6 *vs.* 5.6), perhaps due to the high estimation error obtained as a result of the number of data analyzed and the high coefficient of variation of the trait: 72.3%. Embryo induction was also obtained using cut seeds cultured on EIM2, for other grape cultivars (data not shown).

Germination

In the present work, embryos were able to germinate directly in the induction medium (Fig. 1A); nevertheless, in order to rescue individual plants, they were transferred to tubes with basal medium supplemented with GA_3. After three months of culture in the tubes, 42% of the embryos had developed into normal plants that could be transferred to soil, 32% of the plants showed abnormal development (without an apex or with abnormal leaves), and 12% had only one cotyledon and did not develop into plants (Fig. 1D). The remaining embryos (14%) suffered a delay in germination. A good yield of embryos was obtained from seeds after three to five months of initial culture.

Virus analysis in plants developed from embryos

No amplification of the GLRaV-1 and GLRaV-3 viruses was observed, neither in plants derived from embryos and cultured *in vitro* nor in acclimatized plants

Table 1. Least square mean and standard error for the percentages of explants which had died, were non-responding, or had calli after five months of culture

	Dead	Non-responding	Calli
Ovary stage			
Unopened inflorescences	49.1 ± 1.8 [a]	16.9 ± 2.8 [a]	34.0 ± 6.1 [a]
Opened inflorescences	8.5 ± 1.8 [b]	29.8 ± 2.6 [b]	61.4 ± 5.9 [b]
Fecundated ovaries	2.8 ± 1.4 [c]	28.8 ± 2.5 [b]	66.9 ± 4.8 [b]
Seeds	0.3 ± 0.2 [c]	35.4 ± 2.0 [b]	42.7 ± 4.1 [a]
Medium			
EIM1 (Nitsch)	15.1 ± 1.0 [a]	16.5 ± 3.3 [a]	61.9 ± 3.6 [a]
EIM2 (Mc Woody)	15.2 ± 1.1 [a]	38.3 ± 3.5 [b]	40.1 ± 3.8 [b]
Cutting			
Yes	14.9 ± 1.1 [a]	17.1 ± 3.5 [a]	54.1 ± 3.8 [a]
No	15.3 ± 1.1 [a]	37.8 ± 3.4 [b]	48.4 ± 3.7 [a]

Values with different letters in a column differ significantly ($p < 0.05$).

Table 2. Least square mean and standard error for the percentage of total embryos, direct embryos (in parentheses are the percentages with respect to the total embryos), and number of embryos per seed obtained from seeds cut and cultured on EIM1 and EIM2 media

Medium	Embryos	Direct embryos	Embryos/seed
EIM1 (Nitsch)	64.6 ± 4.4 [a]	48.5 ± 5.3 (75% [a])	4.6 ± 1.0 [a]
EIM2 (McCown Woody)	48.5 ± 4.4 [b]	41.7 ± 5.6 (86% [b])	5.6 ± 1.1 [a]

Values with different letters in a column differ significantly ($p < 0.05$).

Figure 1. Direct (A) and indirect (B) embryogenesis from seeds cut transversally, after 3 months of culture on EIM2 and 1 month in MW medium. C, detail of germinated embryos. D, detail of embryos around a callus (1X). E, germinated embryos of GN cultivar 2 months after sowing on tubes.

grown in the greenhouse. Thus, virus-free plants were obtained from cultivar GN.

Discussion

Amplification of the GLRaV-1 and GLRaV-3 viruses was obtained in cultivar GN. The infection of this old cultivar with GLRaV-1 and GLRaV-3 is in accordance with previous results, since a high level of incidence of these viruses was detected in Alicante province in other table grapes cultivars (Bertolini *et al.*, 2010).

In order to sanitize this material, somatic embryogenesis, which has already proved effective in the elimination of important phloem-limited viruses in grape (Goussard *et al.,* 1991; Gambino *et al.,* 2006), is used. The lack of vascular connection between grapevine somatic embryos and the parent tissue (Newton & Goussard, 1990) can be considered as the reason for the sanitation occurring during the cultures.

Embryogenic competence is highly dependent on the genotype, explant type, and medium composition (Dhekney *et al.*, 2009; Prado *et al.*, 2010). In the present work four different types of explant (cut and uncut) were cultured on EIM1 and EIM2 media, namely unopened and opened inflorescences, fecundated ovaries, and seeds – the latter have not been used before for the induction of embryogenesis in grape. The explants commonly used for grape embryogenesis induction are anthers and ovaries (Goussard *et al.*, 1991; Gribaudo *et al.*, 2004; Kikkert *et al.*, 2005; López-Pérez *et al.*, 2005; Gambino *et al.*, 2006, 2009; Borroto-Fernández *et al.*, 2009). We considered it interesting to test the effect of cutting the explants, so that the medium was in contact with the internal part of the explants. With respect to the culture media, we used the growth regulator TDZ (at 0.9 µM) to induce the embryogenesis in two media that differed in their salt and vitamin composition. This growth regulator was used in the present work since it may increase direct organogenesis and embryogenesis (Gisbert *et al.*, 2006; Chhabra *et al.*, 2008; Ma *et al.*, 2011). Our results show that the salts and vitamins also influenced callus formation, because a higher percentage of direct embryogenesis was obtained in EIM2 compared to EIM1.

After five months of culture, a high amount of embryos was obtained from cut seeds. The fact that embryos were not observed at this time in the rest of the explants may be due to the short period of culture. For instance, somatic embryos from the anthers and ovaries of plants infected with GFLV were sampled by Gambino *et al.* (2009) 6 to 8 months after culture initiation.

Different authors have reported low conversion of grape embryos into normal plants due to morphological alterations or dormancy (Rajasekaran & Mullins, 1979; Goebel-Tourand *et al.*, 1993; Faure *et al.*, 1998; López-Pérez *et al.*, 2006). In the present work, the embryos were able to germinate directly in the induction medium; nevertheless, in order to rescue individual plants, they were transferred to tubes with basal medium supplemented with GA_3. The inclusion of GA_3 was reported to have a positive effect on plant germination in several studies (Rajasekaran & Mullins, 1979; López-Pérez *et al.*, 2006). The percentage of well-developed plants was similar to those reported by Rajasekaran & Mullins (1979) and López-Pérez *et al*. (2006).

A good yield of embryos was obtained from the seeds in the three to five months of initial culture. This period is shorter than others reported for the sanitation of virus-infected grape varieties (Gambino *et al.*, 2006, 2009, 2011; Borroto-Fernández *et al.*, 2009).

The procedure described could be used to induce embryos in other grape varieties and for micropropagation. In the latter case, it could be convenient to test whether somaclonal variation (Larkin & Scowcroft, 1981) was produced in the regenerated plants as a consequence of the culture, as true-to-type regeneration is needed in micropropagation. Molecular markers like AFLP (Vos *et al.*, 1995) and the new tools for epigenetic characterization (Miguel & Marum, 2011) could be used to analyze the plants. We have achieved 86% direct regeneration in EIM2 and obtained apparently normal plants in a short period of culture. Both factors have been described as convenient, to reduce the occurrence of somaclonal variation (Cote *et al*., 2001; Sharma *et al*., 2007; Paul *et al.*, 2011).

This is the first report that describes the use of mature seeds as explants for embryogenesis induction in grape. This type of explant has the advantage that it can be collected and kept for a long time. In addition, this procedure reduces the time needed to regenerate healthy plants, in comparison with other protocols. The new protocol has been very useful for sanitation of the old cultivar GN and could be applied to other interesting grape cultivars. The somatic embryogenesis procedure provided a good yield and it could be used also for micropropagation.

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