GRAPEVINE FLECK VIRUS (GFKV) ELIMINATION IN A SELECTED CLONE OF *VITIS VINIFERA* L. CV. MANTO NEGRO AND ITS EFFECTS ON PHOTOSYNTHESIS

Josefina BOTA[1*], Enrico CRETAZZO[2], Rafael MONTERO[1], Joan ROSSELLÓ[3] and Josep CIFRE[3]

1: Institut de Recerca i Formació Agrària i Pesquera (IRFAP), Conselleria d'Agricultura, Medi Ambient i Territori, Govern de les Illes Balears, C/ Eusebi Estaba, 145, 07009 Palma de Mallorca, Spain
2: Instituto de Investigación y Formación Agraria y Pesquera (IFAPA), centro de Churriana. Consejería de Agricultura, Pesca y Medio Ambiente. Junta de Andalucía. Cortijo de la Cruz s/n, 29140 Málaga, Spain
3: Universitat de les Illes Balears (UIB), Grup de Recerca en Biologia de les Plantes en Condicions Mediterrànies, Cra. Valldemossa km 7.5, 07122 Palma de Mallorca, Spain

Abstract

Aims: The use of healthy propagating material is required to control grapevine viruses. The aim of this work was to eliminate *Grapevine fleck virus* (GFkV) from a Manto Negro clone, a local grapevine variety, in order to include this material in certification programs. Additionally, the effects of virus elimination on photosynthesis and related parameters were evaluated.

Methods and results: Two method combinations for virus elimination were evaluated: (1) field thermotherapy and shoot tip culture and (2) chamber thermotherapy and shoot tip culture. GFkV elimination was tested by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) and reverse transcription-polymerase chain reaction (RT-PCR). The results suggest that a natural field thermotherapy prior to shoot tip culture is effective, making unnecessary the chamber thermotherapy treatment. Additionally, the effects of virus elimination on gas exchanges, chlorophyll fluorescence, electron transport rate (ETR), protein and pigment content were evaluated. The results indicate that GFkV infection affects physiological processes, especially stomatal conductance (g_s), whereas photosynthesis, protein, pigment content, ETR, and fluorescence parameters were not significantly changed.

Conclusion: This study described a simple and rapid method that requires only one medium for virus elimination (GFkV). Beyond its sanitation potential, the use of larger explants (1-3 mm) ensures the integrity of the clone. The presence of the virus affects physiological processes, especially g_s, demonstrating the beneficial effect of eliminating GFkV.

Significance and impact of the study: The described method has the potential to produce GFkV-free rooted plantlets faster than other methods while being potentially safer in maintaining the genetic and phenotypic stability of the regenerated clone. The beneficial effects of GFkV elimination provide evidence for the importance to detect this virus prior to the inclusion of clones in certification programs.

Key words: grapevine, *Grapevine fleck virus* (GFkV), virus eradication, shoot tip culture, gas exchange, chlorophyll fluorescence

Résumé

Objectifs : L'utilisation de matériel de propagation sain est nécessaire pour contrôler les virus de la vigne. L'objectif de ce travail était d'éliminer le *virus de la marbrure de la vigne* (GFkV) d'un clone de cépage local, Manto Negro, dans le but d'inclure ce matériel dans les programmes de certification. De plus, on a pu évaluer les effets de l'élimination du virus sur la photosynthèse et les paramètres qui s'y rapportent.

Méthodes et résultats : Deux combinaisons de méthodes ont été utilisées pour éliminer le virus : (1) la hausse de la température de la vigne combinée à la culture d'apex et (2) la thermothérapie dans une chambre de croissance combinée à la culture d'apex. L'élimination du virus GFkV a été testée par les techniques DAS-ELISA (*double antibody sandwich-enzyme-linked immunosorbent assay*) et RT-PCR (*reverse transcription-polymerase chain reaction*). Les résultats indiquent que le traitement de thermothérapie peut être évité si le matériel végétal est collecté et que les cultures ont été établies à une période très spécifique de l'année. De plus, les effets de l'élimination du virus sur les échanges gazeux, la fluorescence de la chlorophylle, le taux de transport des électrons (ETR) et le contenu en protéines et pigments ont été évalués. Les résultats suggèrent que la présence du virus GFkV affecte les processus physiologiques, en particulier la conductance stomatique (g_s).

Conclusion : Notre étude a décrit une méthode simple et rapide qui ne nécessite qu'un seul milieu pour éliminer le virus (GFkV). Le choix d'explants de taille plus importante (1-3 mm) ainsi que la présence d'une température naturellement élevée dans la région représentent une garantie pour maintenir l'intégrité du clone et obtenir un matériel végétal sain. La présence du virus affecte les processus physiologiques, et plus spécialement la g_s, ce qui démontre l'impact bénéfique de l'élimination de GFkV.

Signification et impact de l'étude : La méthode décrite a le potentiel de produire des plants racinés GFkV-sains rapidement. Elle est plus sûre en comparaison avec d'autres méthodes pour maintenir la stabilité génétique et phénotypique du clone régénéré. Les effets bénéfiques de l'élimination de GFkV mettent en évidence l'importance d'inclure la détection systématique de ce virus dans les programmes de certification.

Mots clés : vigne, *virus de la marbrure de la vigne* (GFkV), éradication du virus, culture d'apex, échanges gazeux, fluorescence de la chlorophylle

manuscript received 30th June 2012 - revised manuscript received 10th January 2014

*Corresponding author : jmbota@dgagric.caib.es

ABBREVIATIONS

BA, 6-benzyladenine; DAS-ELISA, double antibody sandwich-enzyme-linked immunosorbent assay; ETR, electron transport rate; GFkV, *Grapevine fleck virus*; GFLV, *Grapevine fanleaf virus*; GLRaV-1 and -3, *Grapevine leafroll-associated virus 1* and *3*; MS, Murashige and Skoog's basal medium; RH, relative humidity; RT-PCR, reverse transcription-polymerase chain reaction; g_s, stomatal conductance

INTRODUCTION

Manto Negro is the most widely cultivated variety in Majorca (Spain) with approx. 20 % of the total grape cultivation area. The importance of this variety in the Balearic Islands has stimulated a clonal selection process for its characterization, improvement and conservation. Clonal selection is the most common method to improve grapevine varieties (Walter and Martelli, 1997; Mannini, 2000), and usually the genetic and sanitary selections are performed simultaneously (Mannini, 2000). Previous studies have demonstrated that the phytosanitary status of vineyards in the Balearic Islands is strongly degraded due to the high degree of virus infection. Cretazzo *et al*. (2010b) showed that only 6.4% of over 200 Manto Negro vines, sited in 33 different vineyards, were virus-free. Among the candidate clones chosen for evaluation in homologation fields, only one Manto Negro vine displayed the «virus-free certified» status. No other «virus-free certified» vines could be selected as candidate clone because they did not meet the minimum agronomic requirements. Infection by multiple virus strains was detected in 58.4 % of Manto Negro vines. Given this scenario, it was very difficult to identify virus-free clones with suitable agronomic characteristics to be considered as a reference for the grape market.

The final aim of any clonal selection process is the achievement of clones free from the most harmful grapevine viruses (certified clones) while possessing varietal identity. According to the Commission Directive 2005/43/EC amending the Annexes to Council Directive 68/193/EEC on the marketing of grapevine propagation material, each member state should ensure the absence of *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1 and GLRaV-3), *Arabis mosaic virus* (ArMV) and *Grapevine fleck virus* (GFkV) (for rootstocks only) in grapevine nursery plants. A candidate clone, MPL15.01, met the minimum agronomic requirements but was infected with GFkV, a phloem-limited virus responsible for grapevine fleck, a widespread graft-transmissible disease of grapevine (Martelli, 1993). The use of healthy propagating material is strongly advised and represents an important measure to control grapevine viruses, since there is no treatment or cure for viral diseases established in the field. In endemic areas, sanitation becomes the main focus of the clonal selection process. High infection degrees among selected autochthonous clones entail the need for a quick and easy technique in order to "clean up" large amounts of material. Although certification regulations require the absence of GFkV in rootstocks only, it is inappropriate to include a clone infected with GFkV in a certification program. In fact, GFkV can easily move between scion and rootstock through phloem and may negatively affect yield and grape quality, usually in combination with other viruses (Teray, 1990; Kovacs *et al*., 2001; Komar *et al*., 2007; Cretazzo *et al*., 2010a). Despite these findings, there are very few studies on the elimination of GFkV and even less information about its effects on photosynthesis or other physiological parameters. In general, the consequences of biotic stress on *Vitis vinifera* L. are still far from being completely understood; several studies on the effects of virus and other pathogens on grapevine physiology showed highly variable patterns (Balachandran *et al*., 1997; González *et al*., 1997; Christov *et al*., 2001; Sampol *et al*., 2003; Petit *et al*., 2006; Komar *et al*., 2007; Christov *et al*., 2007; Basso *et al*., 2010). In particular, a number of viruses are known to affect grape yield and quality (González *et al*., 1997; Guidoni *et al*., 1997; Cabaleiro *et al*., 1999; Cretazzo *et al*., 2010a; Lee and Martin, 2009; Lee *et al*., 2009; Basso *et al*., 2010). However, there is only scarce information about the effects of GFkV on plant physiology.

The aim of this study was to develop an effective procedure to eliminate GFkV in order to obtain clones suitable for certification. Additionally, the effects of virus elimination on different photosynthetic parameters were evaluated.

MATERIALS AND METHODS

1. Plant material and treatments

A candidate clone (MPL15.01) of Manto Negro cv. was tested for GFLV, GLRaV-1, GLRaV-2, GLRaV-3 and GFkV. It displayed a single infection with GFkV (Cretazzo *et al*., 2010b). Two sanitation methodologies were compared: (1) field thermotherapy and shoot tip culture and (2) chamber thermotherapy and shoot tip culture. Both thermotherapy treatments occurred *in vivo*. In (1), the naturally high and increasing temperature in the vineyard during the experiment acted as a natural thermotherapy; in (2), the

thermotherapy treatment was applied in a growth chamber. Two different media were tested for the establishment of explants: Murashige and Skoog's basal medium (Murashige and Skoog, 1962), 3 % sucrose and 0.7 % agar without any hormonal supplement (MS-0) and supplemented with 2 mg/L of 6-benzyladenine (MS-BA). The same basal media with half strength of macro and microelements were used as rooting media.

1.1. Field thermotherapy and shoot tip culture

Shoots from clone MPL15.01 grown in field trials were taken at its maximum growth period (July 2009). Plants were well irrigated and some shoots removed, ensuring maximum new shoot growth. High temperatures were predicted through the web site www.wetterzentrale.de/topkarten/fsavneur.html. During the week prior to sampling, the average maximum temperature did not fall below 30 ºC, reaching over 39 ºC three days before sampling. The average night-time temperature for the week before the experiment was 19.7 ºC and did not fall below 16 ºC. Shoots were stripped of leaves and washed with tap water. Growing shoot tips were rinsed in 70 % ethanol for 30 s and soaked for 15 min in an aqueous solution of sodium hypochlorite (1 % active chlorine) plus a few drops of Tween-20. After surface sterilization, the plant material was washed three times in sterile distilled water to remove sterilizing agents. Shoot tips (1-3 mm) were isolated and cultivated on MS-0 and MS-BA. Glass test tubes (150 mm x 24 mm) with 25-mm diameter plastic stoppers (Kap-Uts K25, Bellco) and containing 20 ml of medium were used. Cultures were incubated in a Fitoclima S600PLH chamber (Aralab) at 23 ºC under a 16-h photoperiod and light intensity of 56 µmol e^- m^{-2} s^{-1} provided by white fluorescent tubes. Relative humidity (RH) was around 60%. After 5 weeks of culture, explants were transferred to rooting media.

1.2. Chamber thermotherapy

Plants were obtained by direct rooting of 0.2-m dormant canes from selected field-grown MPL15.01 vines. Plants were maintained in the greenhouse until sprouting. After 5 weeks, plants with actively growing shoots were subjected to chamber thermotherapy. Plants were transferred into a growth chamber at 26 ºC/22 ºC (day/night) under a 16-h photoperiod and light intensity of 56 µmol e^- m^{-2} s^{-1} provided by white fluorescent tubes. Temperature was gradually increased by 4 ºC per week. Final day/night temperatures (37.5 ºC/34.0 ºC) were maintained for 40 days. After this period, apical and axillary buds were excised, surface-sterilized and cultivated as described above.

2. Transfer to *ex vitro* conditions

Rooted plantlets were transferred to sterile soil and maintained in a growth chamber at 25 ºC, low light intensity and 90 % RH for 15 days. Plantlets were transferred into a greenhouse under higher light intensity but still with high RH. RH was gradually decreased to obtain full *ex vitro* acclimation and active growth. Two months later, plants were placed outdoors under a shading mesh to protect them from direct sunlight. Plants were grown outdoors in 5-L pots filled with organic substrate and perlite mixture (3:1). Approximately one year after acclimation, when plants had sufficient lignified material, grapevines were sampled to check their sanitary status. Plants were irrigated daily from May to October as follows: 5 times a week with water and twice a week with 50 % Hoagland solution (Hoagland and Arnon, 1950). Gas exchange measurements were performed during the last week of July as described below.

3. Virus detection

3.1 ELISA

Plants were tested for GFkV before and after treatments. Petiole or leaf samples were extracted and analyzed by indirect DAS-ELISA (Clark and Adams, 1977) to detect the presence of GFkV using a commercial kit (Bioreba) according to manufacturer's instructions.

3.2 RT-PCR

Total RNA was extracted from grapevines (50 mg of phloem scraped from mature canes or leaves) using Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to manufacturer's instructions. First-strand cDNA synthesis was performed using 500 ng of total RNA, 200 units of recombinant Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen Life Technologies), 40 units of RNase inhibitor (RNase Out, Invitrogen Life Technologies), 0.4 mM of dNTPs, and 2 mM of random nonamers (Takara Bio). The mixture for reverse transcriptase (20 µl) was incubated for 50 min at 37 ºC and the reaction was heat-inactivated at 70ºC for 15 min.

GFkV-specific primer pairs GFk V1/F and GFk C1/R (Osman *et al*., 2008) were used for PCR amplification of a 344-bp fragment. The PCR reaction mix (25 µl) contained 2 µl of cDNA, 1 mM of dNTPs, 0.5 mM of each primer and 5 units of Taq polymerase (Takara Taq™, Takara Bio). Thermo-cycling was performed as follows: 30 min at 52 ºC followed by 35 cycles of

94 ºC for 30 s, 58 ºC for 45 s and 72 ºC for 60 s, final extension at 72 ºC for 7 min, and storage at -20 ºC. Finally, 10 ml of amplification product was electrophoresed on a 2 % agarose gel in TBE buffer [90 mM Tris–borate, 2 mM EDTA, pH (8.0)], stained with ethidium bromide, visualized on an UV transilluminator and photographed. Positive and negative GFkV controls were included in the experiment.

4. Gas exchange and chlorophyll fluorescence measurements

GFkV-free plants (sanitized) and GFkV-infected (not sanitized) acclimated plants coming from the GFkV elimination experiment were used to determine the possible effects of GFkV on photosynthetic parameters. Four plants per treatment were selected to perform measurements without distinction between the two techniques used for virus elimination. Leaf gas exchange and chlorophyll a (Chl a) fluorescence were simultaneously measured with an open infrared gas exchange analyser system equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor, Lincoln NE, USA). All measurements were taken between noon and 1 P.M. local time at 1500 µmol e^{-} m^{-2} s^{-1}, with a CO_2 concentration in the leaf cuvette of 400 µmol CO_2 mol^{-1} air. Five to eight young, fully expanded leaves from four different plants per treatment were analyzed.

5. Pigment analysis and protein content

Pigment analysis and protein content were performed in eight samples per treatment (two replicates per plant). Leaf discs (5.3 cm^2) were freeze-clamped in liquid nitrogen and stored at -70 °C until assay. Samples were ground to fine powder in a liquid nitrogen-chilled mortar and homogenised in 1 ml of extraction medium. The extraction medium was optimised for this cultivar by testing different components. The extraction medium used contained 0.5 M Tris (pH 7.5), 10 mM EDTA, 1% Triton X-100 and 2 % β-Mercaptoethanol. For pigment determinations, 50 µl of crude extract were diluted in 950 µl of ethanol and after 10 min in the dark, extracts were clarified by centrifugation (12000 rpm at 4 ºC for 2 min) and Chl a, Chl b and carotenoids were quantitatively determined by spectrophotometry according to Lichtenthaler and Wellburn (1983). For protein content determination, extracts were clarified by centrifugation (12000 rpm at 4 ºC for 2 min) and supernatants assayed for total soluble protein according to the method of Bradford (1976).

6. Determination of leaf number and leaf area

Total number of leaves per plant and total leaf area were determined during the same week as gas exchange and chlorophyll fluorescence measurements. Leaf area was measured using public domain image analysis software (ImageJ version 1.32; http://rsb.info.nih.gov/ij/). Twenty leaves of different sizes were used to develop a regression equation for leaf area estimation based on linear measurements (leaf length).

7. Statistical analysis

The influence of media and treatments on survival and virus eradication rates was analyzed by a Chi-Square Test using the SPSS statistical package v12 (SPSS Inc., Chicago IL, USA). One-way ANOVA was also performed for physiological parameters to compare GFkV-free and GFkV-infected plants.

RESULTS

1. Virus detection

The candidate clone MPL15.01 displayed an infection by GFkV (Cretazzo *et al*., 2010b). Virus detection in mother plants grown in a commercial vineyard was carried out by DAS-ELISA. Neither leaf nor other symptoms were observed in these plants, as GFkV is symptomless in *Vitis vinifera* L. Virus infection of mother plants was confirmed by RT-PCR. The expected 344-bp amplification product was observed in GFkV-infected plants (mother and non-sanitized plants), while no amplification was obtained in GFkV-free ones (certified GFkV-free clone used as negative control and sanitized plants) (Figures 1 and 2).

Table 1. Survival rates and virus elimination post "field thermotherapy and shoot tip culture" and "chamber thermotherapy and shoot tip culture" treatments.

Treatment	Regenerated plantlets from shoot tips		Virus elimination
	MS-0	MS-BA	
Field thermotherapy	8/24 (33 %)a	4/25 (16 %)b	3/12 (25 %)ns
Chamber thermotherapy	8/21 (38 %)a	2/20 (10 %)b	2/10 (20 %)ns

Different letters mean significant differences (P<0.05); ns, non significant differences at P≥0.05

2. Explant survival and virus elimination

Regeneration rate from shoot tip culture was significantly higher in the MS-0 medium than in the MS-BA medium in both treatments (Table 1). In spite of the efforts for virus elimination using shoot tips instead of meristems, the survival rates were low. Values for shoot tip survival were 33 % in MS-0 and 16 % in MS-BA for the field thermotherapy treatment. Similar values were obtained in plants undergoing heat treatment in a growth chamber (38 % in MS-0 and 10 % in MS-BA). The addition of BA to the medium resulted in considerable shoot proliferation and hyperhydricity, also known as vitrification (Figure 3). These results indicate that BA is not necessary for proliferation and rooting and may even be detrimental.

Based on ELISA tests, GFkV was eliminated from 25 % of the regenerated plantlets in the field thermotherapy and shoot tip culture treatment (Table 1). In order to confirm these results, RT-PCR amplification was carried out for ELISA-negative samples. No PCR products were obtained from these samples, therefore confirming the immunoenzymatic assay results (Figure 1).

The percentage of sanitized plants was not significantly increased when the heat treatment was performed in a growth chamber prior to shoot tip culture, with 20 % of the plants testing negative (Table 1). Figure 2 shows the RT-PCR products obtained from ELISA-negative samples and controls (positive and negative) using specific primers for GFkV. The RT-PCR results confirm the percentage of sanitized plants, as no RNA amplification was obtained in lanes corresponding to ELISA-negative samples coming from the chamber thermotherapy and shoot tip culture treatment.

3. Effects of virus elimination on leaf area and photosynthetic parameters

GFkV-free plants presented fewer but larger leaves than infected ones; therefore total leaf area was not significantly different (Table 2).

3.1. Gas exchange

GFkV-infected plants showed lower photosynthetic rate (A_N), although the difference was not statistically

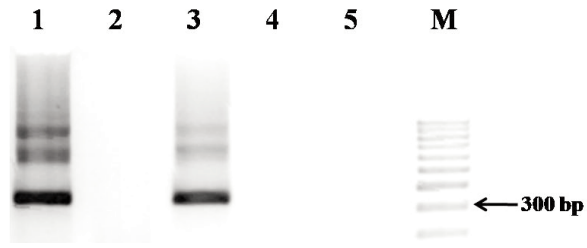

Figure 1. Agarose gel analysis of RT-PCR assays with specific primers for GFkV.
Lane 1. Positive sample originated from field thermotherapy and shoot tip culture (non-sanitized plant). Lane 2. Negative sample originated from field thermotherapy and shoot tip culture (sanitized plant). Lane 3. Field-grown original plant material, positive control. Lane 4. Certified clone, negative control. Lane 5. Blank. M. 100-bp DNA ladder.

Table 2. Total leaf area, leaves per plant, total chlorophyll content (Chl), carotenoid and total soluble protein of leaves, net CO_2 assimilation rate (A_N), stomatal conductance (g_s), leaf transpiration (E), internal CO_2 concentration (Ci), electron transport rate (ETR), maximum fluorescence rate (Fm') and efficiency of PSII (FPSII) in GFkV-free and -infected Manto Negro clones.

Parameter	GFkV-free plants	GFkV-infected plants	% change	P
Total leaf area (m^2)	0.38±0.08	0.50±0.03	+32.58	n.s.
Leaves·plant^{-1}	61.25±11.72	100.33±4.33	+63.81	*
Chl total (g·m^{-2})	0.23±0.01	0.25±0.02	+11.02	n.s.
Carotenoids (g·m^{-2})	0.04±0.00	0.04±0.00	+5.69	n.s.
Soluble protein (g·m^{-2})	1.96±0.23	2.53±0.20	+29.05	n.s.
A_N (µmol CO_2·m^{-2}·s^{-1})	9.59±1.25	7.80±0.64	-18.6	n.s.
g_s (mol HO_2·m^{-2}·s^{-1})	0.12±0.02	0.05±0.01	-57.29	*
E (mmol HO_2·m^{-2}·s^{-1})	3.67±0.42	1.41±0.18	-61.43	**
Ci (µmol CO_2· mol^{-1})	247.75±9.30	136.02±18.18	-45.1	***
ETR (µmol e$^-$·m^{-2}·s^{-1})	94.78±11.65	93.53±4.81	-1.32	n.s.
Fm'	612.56±32.36	656.74±17.78	+7.21	n.s.
ΦPSII	0.15±0.02	0.15±0.01	-1.26	n.s.

Values are means ± S.E., n=4-8 % Change indicates the percentage of change (-, decrease, +, increase) in GFkV-infected plants with respect to GFkV-free plants for each parameter. Statistically significant differences between treatments are indicated by *P<0.05, **P<0.01, and ***P<0.001; n.s., non significant differences.

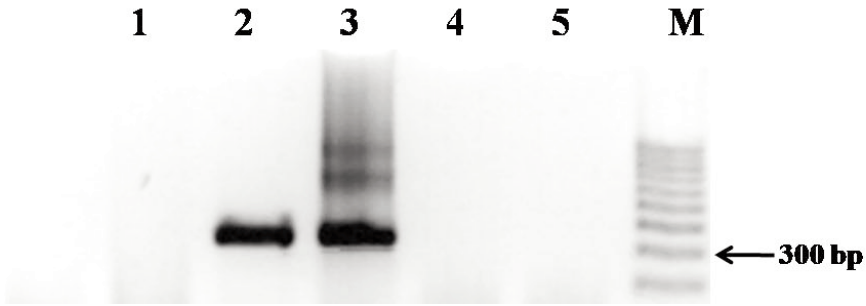

Figure 2. Agarose gel analysis of RT-PCR assays with specific primers for GFkV.
Lane 1. Negative sample originated from chamber thermotherapy and shoot tip culture (sanitized plant). Lane 2. Positive sample originated from chamber thermotherapy and shoot tip culture (non-sanitized plant). Lane 3. Field-grown original plant material, positive control. Lane 4. Certified clone, negative control. Lane 5. Blank. M. 100-bp DNA ladder.

significant (Table 2). However, significant reductions were observed in stomatal conductance (g_s) and transpiration rate (E). Intercellular CO_2 concentration (Ci) was lower in GFkV-infected plants; this decrease indicates that non-stomatal limitations are not present, which is consistent with the absence of variation in protein content (Table 2).

3.2. Chlorophyll fluorescence and pigment content

No differences were observed between GFkV-free and GFkV-infected plants in the chlorophyll fluorescence parameters Fm', ΦPSII and ETR (Table 2). This suggests that PSII was not impaired. There were no visible leaf symptoms in infected plants. Chlorophyll and carotenoid contents did not change with viral infection, which confirms that pigment degradation did not occur by GFkV infection (Table 2).

DISCUSSION

The high incidence of virus infection in Manto Negro cv. requires the elimination of harmful viruses from selected clones using the most appropriate technique. Sanitation is the only way to generate a virus-free clone to be included in clonal selection process.

Several techniques have been used for grapevine virus elimination. Both meristem culture (Sampol *et al*., 2003; Maliogka *et al*., 2009; Youssef *et al*., 2009) and somatic embryogenesis (Gambino *et al*., 2006; Borroto-Fernandez *et al*., 2009; Gambino *et al*., 2009) are reported to eliminate different virus infections from *Vitis vinifera* L. The lack of vascular connections between parent tissue and meristems or embryos can be the reason for the sanitation occurring during cultures, especially for phloem-limited virus like GFkV. To maximize virus elimination, these methods may be combined with heat treatment before or after the establishment of tissue culture (Krizan *et al*., 2009; Gribaudo *et al*., 2006). Valero *et al*. (2003) claimed that a form of "natural thermotherapy" can occur during summer when the vineyard temperatures reach more than 38 ºC. *In vitro* culture of shoot tips under these conditions was used with success for GLRaV-3 and GFLV (Valero *et al*., 2003), but none of these methodologies were tested for GFkV elimination. In the present study, the combination of either high temperature during summer in the field or growth chamber thermotherapy treatment with shoot tip culture was effective for the elimination of GFkV (25 % and 20 %, respectively). This contrasts with Panattoni and Triolo (2010), who recently showed that GFkV was insensitive to heat stress. Komar *et al*. (2007) managed to eliminate GFkV from a clone of

Figure 3. 6-Benzyladenine (BA) effect on shoot proliferation. (A) Explant established on MS-BA medium. (B) Explant established on MS-0 medium.

Chardonnay using only shoot apex culture, however, the elimination rate was not presented. In general, it seems that the success of a method not only depends on grapevine genotype but also on virus species and the specific virus-grapevine interactions (Maliogka *et al*., 2009). It is likely that the application of other elimination techniques such as somatic embryogenesis or meristem culture to GFkV-infected vines will provide higher sanitation rates, as shown for other virus infections (Gambino *et al*., 2006; Gribaudo *et al*., 2006; Borroto-Fernandez *et al*., 2009; Gambino *et al*., 2009). However, these virus elimination techniques are much more difficult, laborious, time consuming and present the risk of genetic variation (Popescu *et al*., 2002).

The possibility of somaclonal variation should also be taken into account. Although genetic changes in the grapevine genome after *in vitro* or thermotherapy treatments are minimal or null (Baránek *et al*., 2009; 2010), the use of larger explants (>1 mm) in the present experiment gives additional security to minimize genetic or phenotypic changes induced by tissue culture. In this sense, a similar method with a reduced subculture number and the use of hormone-free culture medium during growth phase was used in the sanitation of Malvasia de Banyalbufar cv. (Sampol *et al*., 2003) and showed to be efficient in the non-appearance of juvenile characters (Carámbula *et al*., 2009). Therefore, it should be possible to achieve GFkV elimination from grapevine by using relatively large explants (1-3 mm) and avoiding the difficulties of meristem tip culture or somatic embryogenesis. A culture medium without any hormone supplement was effective for proliferation and rooting. The lower survival of plantlets in MS-BA medium could be attributed to vitrification. Vitrified plantlets have high water content in leaf and stem tissues and poor epicuticular wax production (Pâques and Boxus, 1987; Majada *et al*., 2001), which lowers survival under *ex vitro* conditions. The use of a culture medium without hormone supplements really simplifies the production of GFkV-free plants.

GFkV-free and still GFkV-infected plants were compared in order to assess the effects of virus elimination on grapevine physiology. Results clearly showed that GFkV infection induced a significant g_s reduction and lower internal CO_2 concentration. Consequently, lower photosynthesis was observed in GFkV-infected plants, although the reduction was not statistically significant, as observed in other grapevine varieties and virus infections (Guidoni *et al*., 1997; Cabaleiro *et al*., 1999; Sampol *et al*., 2003) or other grapevine pathogens as Esca (Petit *et al*., 2006) and Pierce's disease (Goodwin *et al*., 1988). It seems that the effects are mostly at the stomatal level, since neither fluorescence parameters nor the pigment content or ETR were affected. In contrast to previous suggestions (Sampol *et al*., 2003), the smaller reduction in photosynthesis with respect to g_s suggests that, despite the reduction of CO_2 availability at the substomatal level, Rubisco activity seems unaffected. This is consistent with the non-alteration of total protein content. A possible explanation for the maintenance of photosynthetic activity despite the significant reduction in g_s could be an increase in mesophyll conductance (g_{mes}), which somehow compensates a lower CO_2 input. The lack of co regulation between g_s and g_{mes} has been demonstrated under stress conditions (Perez-Martin *et al*., 2009). This hypothesis disagrees with findings in Malvasia de Banyalbufar vines infected by GFLV and GLRaV (Sampol *et al*., 2003) since g_{mes} was lower in virus-infected than in non-infected plants and the reduction of CO_2 diffusion through the mesophyll was considered an important factor limiting photosynthesis in virus-infected plants. Genotype, virus type and degree of virus infection may play an essential role in the severity of biotic stress. Further studies with different grape cultivars and/or virus infections will provide a better understanding of the mechanisms of virus-vine interactions.

CONCLUSION

Our study described a simple and rapid method that requires only one medium for virus elimination (GFkV). The method has the potential to produce GFkV-free rooted plantlets in a shorter time than other methods and is potentially safer with respect to maintaining the genetic stability of the regenerated clone. Furthermore, the data presented here suggest that GFkV can affect physiological processes, especially g_s, but further studies on these disturbances are needed to better understand the influence of the virus on photosynthesis.

Acknowledgements: This work was supported through research projects RTA2008-00085-C02-01 and RTA2010-00118-00-00 funded by INIA, Spain. We gratefully acknowledge D. Francis and M. Ribas-Carbó for grammar correction. We are also grateful to C. Padilla for helping in data collection and H. El Aououad for translating the abstract into French.

REFERENCES

Balachandran S., Hurry V.M., Kelley S.E., Osmond C.B., Robinson S.A., Rohozinski J., Seaton G.G.R. and Sims D.A., 1997. Concepts of plant biotic stress. Some insights into the stress physiology of virus-infected plants, from the perspective of photosynthesis. *Physiol. Plant.*, **100**, 203-213.

Baránek M., Raddova J., Krizan B. and Pidra M., 2009. Genetic changes in grapevine genomes after stress induced by *in vitro* cultivation, thermotherapy and virus infection, as revealed by AFLP. *Genet. Mol. Biol.*, **32**, 834-839.

Baránek M., Krizan B., Ondrusikova E. and Pidra M., 2010. DNA-methylation changes in grapevine somaclones following in vitro culture and thermotherapy. *Plant Cell Tissue Organ Cult.*, **101**, 11-22.

Basso M.F., Fajardo T.V.M., Santos H.P., Guerra C.C., Ayub R.A. and Nickel O., 2010. Fisiologia foliar e qualidade enológica da uva em videiras infectadas por vírus. *Trop. Plant Pathol.*, **35**, 351-359.

Borroto-Fernandez E.G., Sommerbauer T., Popowich E., Schartl A. and Laimer M., 2009. Somatic embryogenesis from anthers of the autochthonous *Vitis vinifera* cv. Domina leads to *Arabis mosaic virus*-free plants. *Eur. J. Plant Pathol.*, **124**, 171-174.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

Cabaleiro C., Segura A. and Garcia-Berrios J.J., 1999. Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *Am. J. Enol. Vitic.*, **50**, 40-44.

Carámbula C., Bota J. and Medrano H., 2009. Recuperación, saneamiento y evaluación en condiciones de Campo de la "Malvasía de Banyalbufar". In: *Proceedings of the 3rd International Symposium "Malvasias"*, La Palma (Spain), paper 4, pp. 1-6.

Christov I.K., Stefanov D., Goltsev V.N. and Abrasheva P., 2001. Effects of grapevine fanleaf and stem pitting viruses on the photosynthetic activity of grapevine plants grown *in vitro*. *Russ. J. Plant Physiol.*, **48**, 473-477.

Christov I., Stefanov D., Velinov T., Goltsev V., Georgieva K., Abracheva P., Genova Y. and Christov N., 2007. The symptomless leaf infection with grapevine leafroll associated virus 3 in grown in vitro plants as a simple model system for investigation of viral effects on photosynthesis. *J. Plant Physiol.*, **164**, 1124-1133.

Clark M.F. and Adams A.N., 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.*, **34**, 475-483.

Cretazzo E., Padilla C., Carambula C., Hita I., Salmeron E. and Cifre J., 2010a. Comparison of the effects of different virus infections on performance of three Majorcan grapevine cultivars in field conditions. *Ann. Appl. Biol.*, **156**, 1-12.

Cretazzo E., Tomás M., Padilla C., Rosselló J., Medrano H., Padilla V. and Cifre J., 2010b. Incidence of virus infection in old vineyards of local grapevine varieties from Majorca: implications for clonal selection strategies. *Span. J. Agric. Res.*, **8**, 409-418.

Gambino G., Bondaz J. and Gribaudo I., 2006. Detection and elimination of viruses in callus, somatic embryos and regenerated plantlets of grapevine. *Eur. J. Plant Pathol.*, **114**, 397-404.

Gambino G., Di Matteo D. and Gribaudo I., 2009. Elimination of *Grapevine fanleaf virus* from three *Vitis vinifera* cultivars by somatic embryogenesis. *Eur. J. Plant Pathol.*, **123**, 57-60.

González E., Mosquera M.V., San José M.C. and Díaz T., 1997. Influence of virus on the chlorophyll, carotenoid and polyamide contents in grapevine microcuttings. *J. Phytopathol.*, **145**, 185-187.

Goodwin P.H., DeVay J.E. and Meredith C.P., 1988. Physiological responses of *Vitis vinifera* cv. 'Chardonnay' to infection by the Pierce's disease bacterium. *Physiol. Mol. Plant Pathol.*, **32**, 17-32.

Gribaudo I., Gambino G., Cuozzo D. and Mannini F., 2006. Attempts to eliminate *Grapevine rupestris stem pitting-associated virus* from grapevine clones. *J. Plant Pathol.*, **88**, 293-298.

Guidoni S., Mannini F., Ferrandino A., Argamante N. and Di Stefano R., 1997. The effect of grapevine leafroll and rugose wood sanitation on agronomic performance and berry and leaf phenolic content of a Nebbiolo clone (*Vitis vinifera* L.). *Am. J. Enol. Vitic.*, **48**, 438-442.

Hoagland D.R. and Arnon D.I., 1950. *The Water-Culture Method for Growing Plants Without Soil*. California Agricultural Experiment Station, Circular no. 347.

Komar V., Vigne E., Demangeat G. and Fuchs M., 2007. Beneficial effect of selective virus elimination on the performance of *Vitis vinifera* cv. Chardonnay. *Am. J. Enol. Vitic.*, **58**, 202-210.

Kovacs L.G., Hanami H., Fortenberry M. and Kaps M.L., 2001. Latent infection by leafroll agent GLRaV-3 is linked to lower fruit quality in French–American hybrid grapevines Vidal blanc and St. Vincent. *Am. J. Enol. Vitic.*, **52**, 254-259.

Krizan B., Ondrusikova E., Holleinova V., Moravcova K. and Blahova L., 2009. Elimination of *Grapevine fanleaf virus* in grapevine by *in vivo* and *in vitro* thermotherapy. *Hort. Sci.*, **36**, 105-108.

Lee J., Keller K.E., Rennaker C. and Martin R.R., 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir: Free amino acids, sugars, and organic acids. *Food Chem.*, **117**, 99-105.

Lee J. and Martin R.R., 2009. Influence of grapevine leafroll associated viruses (GLRaV-2 and -3) on the fruit composition of Oregon *Vitis vinifera* L. cv. Pinot noir: Phenolics. *Food Chem.*, **112**, 889-896.

Lichtenthaler H.K. and Wellburn A.R., 1983. Determinations of total carotenoids and chlorophylls a

and b of leaf extracts in different solvents. *Biochem. Soc. Trans.*, **11**, 591-592.

Majada J.P., Sierra M.I. and Sanchez-Tames R., 2001. Air exchange rate affects the in vitro developed leaf cuticle of carnation. *Sci. Hortic.*, **87**, 121-130.

Maliogka V.I., Skiada F.G., Eleftheriou E.P. and Katis N.I., 2009. Elimination of a new ampelovirus (GLRaV-Pr) and *Grapevine rupestris stem pitting associated virus* (GRSPaV) from two *Vitis vinifera* cultivars combining *in vitro* thermotherapy with shoot tip culture. *Sci. Hortic.*, **123**, 280-282.

Mannini F., 2000. Clonal selection in grapevine: interactions between genetic and sanitary strategies to improve propagation material. *Acta Hort.*, **528**, 703-712.

Martelli G.P., 1993. *Graft-Transmissible Diseases of Grapevines: Handbook for Detection and Diagnosis.* FAO Publication Division, Rome, Italy.

Murashige T. and Skoog F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473-497.

Osman F., Leutenegger C., Golino D. and Rowhani A., 2008. Comparison of low-density arrays, RT-PCR and real-time TaqMan® RT-PCR in detection of grapevine viruses. *J. Virol. Methods*, **149**, 292-299.

Panattoni A. and Triolo E., 2010. Susceptibility of grapevine viruses to thermotherapy on *in vitro* collection of Kober 5BB. *Sci. Hortic.*, **125**, 63-67.

Pâques M. and Boxus P., 1987. "Vitrification": review of literature. *Acta Hort.*, **212**, 155-166.

Perez-Martin A., Flexas J., Ribas-Carbó M., Bota J., Tomàs M., Infante J.M. and Dıaz-Espejo A., 2009. Interactive effects of soil water deficit and air vapour pressure deficit on mesophyll conductance to CO_2 in *Vitis vinifera* and *Olea europaea*. *J. Exp. Bot.*, **60**, 2391-2405.

Petit A.N., Vaillant N., Boulay M., Clément C. and Fontaine F., 2006. Alteration of photosynthesis in grapevines affected by Esca. *Phytopathology*, **96**, 1060-1066.

Popescu C.F., Falk A. and Glimelius K., 2002. Application of AFLPs to characterize somaclonal variation in anther-derived grapevines. *Vitis*, **41**, 177-182.

Sampol B., Bota J., Riera D., Medrano H. and Flexas J., 2003. Analysis of the virus-induced inhibition of photosynthesis in malmsey grapevines. *New Phytol.*, **160**, 403-412.

Teray Y., 1990. Anjinashika disease: a combined effect of grapevine leafroll and grapevine fleck viruses on sugar content in the Japanese grape cultivar Koshu. In: *Proceedings of the 10th Meeting of ICVG*, Volos (Greece), pp. 67-70.

Valero M., Ibanez A. and Morte A., 2003. Effects of high vineyard temperatures on the grapevine leafroll associated virus elimination from *Vitis vinifera* L. cv. Napoleon tissue cultures. *Sci. Hortic.*, **97**, 289-296.

Walter B. and Martelli G.P., 1997. Sélection clonale de la vigne : sélection sanitaire et sélection pomologique. Influence des viroses et qualité. 2e partie : sélection sanitaire, sélection pomologique. *Bull. OIV*, **70**, 5-23.

Youssef S.A., Al-Dhaher M.M.A. and Shalaby A.A., 2009. Elimination of Grapevine fanleaf virus (GFLV) and Grapevine leaf roll-associated virus-1 (GLRaV-1) from infected grapevine plants using meristem tip culture. *Int. J. Virol.*, **5**, 89-99.