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High prevalence of viruses in table grape from Spain detected by real-time RT-PCR

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Abstract Table grapes from one of the most important growing area in Spain (Vinalopó, Alicante) protected by the Designation of Origin "Vinalopó bagged table grape", were surveyed and analysed to determine the prevalence of the five viruses included in the Spanish certification program: *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus-1* (GLRaV-1) and *Grapevine leafroll associated virus-3* (GLRaV-3). Ninety five sampling points were selected and the position of grapevine plants georeferenced. Samples were collected in two different vegetative periods and analyses were performed by ELISA and real-time RT-PCR. Purified RNA and immobilized viral targets from plant extracts on nylon membranes were used in parallel assays as templates for PCR assays. In order to analyse these five viral species by real-time RT-PCR, new specific primers and TaqMan probes were designed for detection of ArMV and GFkV. Real time RT-PCR from purified RNA was more sensitive than spot version and ELISA tests. The most prevalent virus was GFLV (95.8%) followed by GLRaV-3 (94.7%), GLRaV-1 (66.3%) and GFkV (65.3%). ArMV was not detected in any sample. The high level of viral infections and the presence of mixed infections suggest that initial infected plant material and uncontrolled traffic of propagation material have played an important role in the spread of viruses.

Keywords ELISA · Spot real-time RT-PCR · Grapevine viruses

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Approximately 7,500 ha of grape (*Vitis* spp.) are grown in the middle valley of the river Vinalopó, the west central region of Alicante province (Spain). The production of this region is mainly focused on table grape cultivars, Ideal and Aledo that are protected by the Designation of Origin "Vinalopó bagged table grape". The protected grapes come exclusively into the "Extra" and "1st Class" categories of the EC Regulation 1730/87. The general sanitary status of the grapevines grown in this important producing area is unknown in spite of the presence of suspicious viral symptoms and the marked decreasing of the vineyards production after four or five years since they were planted. Traditional diagnosis of viruses that infect

grapevine is based on biological indexing using woody indicators and herbaceous hosts, along with enzyme-linked immunosorbent assay (ELISA). However, biological indexing is a high cost technique with problems for large-scale analysis that requires a long time to confirm infection by visual symptoms. Serological tests overcome these disadvantages, but the major limitations are the inability to detect grapevine viruses present in plants at low titre mainly using polyclonal antibodies. For these reasons rapid techniques with high sensitivity and specificity are required in order to implement phytosanitary certification schemes to control viral diseases. Polymerase chain reaction (PCR) modified the scene of molecular diagnosis, holding the promise for an improved management and control of infectious diseases. Nevertheless, conventional PCR-based methods require an additional step to detect specific amplicons, gel electrophoresis or hybridisation. And thus, such steps increase risks of contaminations giving false positive results. On the other hand, false negative results from PCR inhibitions due to plant compounds prevent the use of conventional PCR-based methods as a high throughput technique. In order to increase sensitivity and accuracy, real-time RT-PCR approaches have been developed and successfully assayed for the detection of viral targets in woody plant material and vectors overcoming many of the drawbacks of conventional PCR-based methods (Capote et al. 2009; Osman and Rowhani 2006; Olmos et al. 2005). In this study, serological tests, routinely used in sanitary selection and certification programs, based on commercially available DAS-ELISA and DASI-ELISA were compared with real-time RT-PCR to assess the prevalence of five grapevine viruses (ArMV, GFLV, GFkV GLRaV-1 and GLRaV-3), since certified material must be free from these agents under European Directive 2002/11/CE, in table grape grown in Alicante protected by the Designation of Origin "Vinalopó bagged table grape".

Viral isolates of each tested virus species kept in a collection under screenhouse at IVIA were used as positive controls. Simple random sampling based on data of planting area with 99% confidence interval (Lohr 2000) was applied to select sampling points and to evaluate prevalence of viruses in this region. A total of 95 plants were selected, georeferenced with a GPS receiver, labelled and analysed. Samples were collected in February (winter) and also in May (spring) from Agost, Aspe, Elx, Hondón de las Nieves, Hondón de los Frailes, Monforte del Cid, Monóver, Novelda, Orihuela and La Romana municipalities. Sampling was performed collecting at least five dormant buds or bark tissues, shoots or complete spurs (winter) or five fully expanded leaves (spring) around the grapevine plant. Extracts were prepared from leaves or cambial scrapping cuttings by grinding aprox. 1/20 (*w/v*) in PBS buffer, pH 7.2, supplemented with 0,2% (*w/v*) DIECA, and 2% (*w/v*) polyvinil-pyrrolidone (PVP-10) in individual plastic bags with a net (Plant Print Diagnostics) to avoid contaminations among samples. The same crude extracts were used for ELISA tests, for spotting procedure and for total RNA purification. For conventional real-time RT-PCR, total RNA was extracted from 200 μl of crude extracts using the RNeasy plant mini kit (Qiagen) or Ultraclean Plant RNA isolation kit (MoBio). The spot procedure was carried out loading 5 μl of plant crude extract onto 0.45-μm positively charged nylon membrane (Olmos et al. 1996) and viral targets were extracted with glycine buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA, pH 8.0) (Osman and Rowhani 2006).

Double antibody sandwich (DAS) ELISA (ArMV, GFLV, GLRaV-1, GLRaV-3) and double antibody sandwich indirect (DASI) ELISA (GFkV), were performed according to instructions of the manufacturer (Agritest), using PBS buffer (pH 7.2) supplemented with 0.2% (*w/v*) DIECA and 2% (*w/v*) polyvinil-pyrrolidone (PVP-10) as extraction buffer.

New specific primers and probes were designed for ArMV and GFkV detection from the coat protein and replicase regions respectively. Sequenced regions of each virus were recovered using the Nucleotide Sequence Search program (Entrez Browser; National Center for Biotechnology Information). Conserved regions for each virus were identified using the similarity search tool Advanced BLAST 2.0, using the blastn program designed to support analysis of nucleotides (Altschul et al. 1997). The alignment view was carried out as master-slave with identities, to analyse significant nucleotide homologies in the molecular data retrieved from NCBI's integrated databases, GenBank, EMBL and DDBJ. Specific nucleotide regions were selected. Finally specific criteria set by Primer Express software (Applied Biosystems) were followed to design primers and probes. In the case of ArMV, the size of the

amplification product was 203 bp and two different probes were included due to the high variability of this virus. The design of the first ArMV probe was based on a highly conserved region of all isolates included to date in the GenBank database. An additional second probe was included for the successful detection of some viral isolates of ArMV recently detected in Spain (Abelleira et al. 2010) that showed 85% nucleotide sequence indentity with NW isolate (Accession No. AY017339). On the other hand the design of specific primers and one TaqMan probe based on all isolates included in GenBank database allowed the amplification of a 146 bp product and the specific detection of all tested GFkV isolates. In the case of GFLV, GLRaV-1 and GLRaV-3 viruses, specific primers and TaqMan probes previously described were used (Osman et al. 2008). Table 1 summarised specific primers and TaqMan probes used in this study. Real-time RT-PCR were performed in a StepOne Plus thermocycler (Applied Biosystems). Final volume of reaction cocktails was 12 µl containing 1 × AgPath-ID One-step RT-PCR master mix (Ambion), 1 × RT-PCR enzyme mix (Ambion), 0.5 µM each primer, 150 nM TaqMan probe and 3 µl of template. Real-time RT-PCR protocol consisted of one step at 45°C for 10 min and 95°C for 10 min followed by 45 cycles of amplification (95°C for 15 s and 60°C for 1 min for GFLV, GFkV, GLRaV-1 and GLRaV-3 detection, and 95°C for 15 s, 50°C for 15 s and 60°C for 45 s for ArMV detection). Data acquisition and analysis were performed with 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.

The most sensitive method for detection of the assayed grapevine viruses was conventional real-time RT-PCR from purified RNA templates, confirming all positive spotted samples and ELISA tests. Spotting procedure facilitated sample preparation for real time assays. However, in all cases this approach resulted in a decrease of sensitivity compared with results of the same RNA purified samples. Although in the dormant period spot real-time RT-PCR was more sensitive than ELISA tests, in the spring the spotting procedure was not useful probably due to the high contents of inhibitors such as phenolic compounds in grapevine material. The results of the analyses are summarised in Table 2. The absence of ArMV in the west central region of Alicante, characterised by a very warm climate, can be explained by the low incidence of this virus in Spain, where only recently has it been reported for the first time and its association to cool-climate viticulture (Abelleira et al. 2010). The high

Table 1 Sequences of primers and probes used for real-time RT-PCR

Virus	Primer/probe[a]	Nucleotide sequence 5′-3′	Reference
ArMV	ArMV i1	AATTATATGCTGAGTTTGAG	Bertolini et al. (2003)
	ArMV i2	AAAATTATACACCTTATGAGTA	
	ArMV p S3	ACCAGTGCCTACAAGAGTGTGTCC	This study
	ArMV p ES	ACCAGTGCTTATAAGAGTGTTTCC	
GFLV	GFLV-769f	GGGACCACTATGGATGGAATGA	Osman and Rowhani (2006)
	GFLV-868r	TTCGGTGATATGGAGAGCGAAT	
	GFLV-799p	AAGTATCCCGGGGTGTATGTGGAAGAGGA	
GFkV	GFkV OB F	CGAGAACTCTCTTTTCACCTC	This study
	GFkV OB R	CCGGCGTGGATGTAGAG	
	GFkV OB S	ACCCTCGCCCTCATGCA	
GLRaV-1	LR1 HSP70-149 f	ACCTGGTTGAACGAGATCGCTT	Osman et al. (2007)
	LR1 HSP70-293 r	GTAAACGGGTGTTCTTCAATTCTCT	
	LR1 HSP70- 225 p	ACGAGATATCTGTGGACGGA	
GLRaV-3	GLRaV3-56f	AAGTGCTCTAGTTAAGGTCAGGAGTGA	Osman and Rowhani (2006)
	GLRaV3-285r	GTATTGGACTACCTTTCGGGAAAAT	
	GLRaV3-181p	CAGGTAATAGCGGACTGAGACTGGTGGACA	

[a] All probes are labelled with 5′-FAM fluorophore and 3′-TAMRA quencher

Table 2 Results of ELISA and real-time RT-PCR tests for the detection of five grapevine viruses. Number of infected plants by detection methods / total of plants analysed

Virus species	Vegetative period					
	Dormant			Growth		
	ELISA	Spot real-time RT-PCR	Conventional real-time RT-PCR	ELISA	Spot real-time RT-PCR	Conventional real-time RT-PCR
GFLV	3/95	12/95	45/95	55/95	10/95	91/95
ArMV	0/95	0/95	0/95	0/95	0/95	0/95
GFkV	25/95	46/95	62/95	48/95	28/95	59/95
GLRaV-1	35/95	57/95	63/95	6/95	6/95	8/95
GLRaV-3	61/95	66/95	90/95	4/95	55/95	67/95

level of incidence of GFLV detected by conventional real-time RT-PCR (95.8%) was dramatically higher than expected. The incidence of mealybug-transmitted viruses, leafroll-associated viruses GLRaV-1 and GLRaV-3 were 66.3% and 94.7% respectively. In the case of GFkV with no vector associated to the transmission the incidence was also very high (65.3%). In fact, the levels of viral infections reported in this study are higher than those described by other authors that used ELISA tests in surveys (Akbas et al. 2009; Fuchs et al. 2009; Kominek 2008). This study appraises critically grapevine virus diagnosis in field samples in several aspects. First, it confirms that the best season to test plant material depends on the virus species due to a higher number of positive results in a specific season but also because of a more close concordance among techniques. Thus, GFLV detection should be performed in the spring because in the dormant period the number of positive detections is severely lower (95.8% vs 47.4% by conventional real-time RT-PCR). Moreover, in spring ELISA tests resulted positive in 55 out of the 91 positive plants given by real-time RT-PCR whilst in winter only 3 grapevine plants gave a positve result by ELISA tests. However, in the case of leafroll associated virus species the recommended vegetative period is during dormancy where the detection is better than in the growth period. And the case of GLRaV-1 detection was rigorously very different in each season (66.3% vs 8.4% by conventional real-time RT-PCR or 36.8% vs 6.3% by ELISA) (see Table 2). The period for detecting GFkV is less critical and although the best detection was performed in dormancy (65.3% by conventional RT-PCR) almost all positive detections could be afforded in spring (59 out of 62 infected grapevine plants). A second assessment focuses on the discordance between results of ELISA tests commonly used in sanitary selection programs and conventional real-time RT-PCR. This suggests the poor sensitivity afforded by ELISA tests and the necessity to improve the reliability of detection methods for sanitary programs. The detection of viruses in grapevine is difficult because there are seasonal variations in the concentration of viruses and ELISA tests are not sensitive enough and reliable in different seasons, which agreed with reports by other authors (Osman et al. 2008). The third consideration is that these high prevalences of viruses cannot be explained only by vector transmission and suggest that initial infected plant material and uncontrolled traffic of propagation material, were key factors for these high levels of infection. Reliable and sensitive methods are extremely useful for detecting infected material and the adoption of conventional real-time RT-PCR in certification programs could improve detection potential, leading to better control of leafroll and grapevine degeneration diseases.

These results led to in depth review of control programs in Vinalopó region. A program to provide virus-free plant material has emerged as a cornerstone of virus control, based on shoot-tip culture of selected clones of Ideal and Aledo cultivars. This is the first report of grapevine virus incidence in the D.O. “Vinalopó bagged table grape” by real-time RT-PCR.

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