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Detection of four regulated grapevine viruses in a qualitative, single tube real-time PCR with melting curve analysis

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ABSTRACT

The detection of the four grapevine viruses (GLRaV-1, GLRaV-3, GFLV and ArMV) regulated in European Union plant material certification, requires sensitive and specific diagnostic tools. A strategy of simultaneous detection in a real-time single tube amplification was developed, based on the EvaGreen binding dye. The melting curve analysis (MCA) of the amplicons allows a qualitative detection of the four different virus targets in multiplex analysis. A plasmid dilution assay calculated an analytical sensitivity with an amplification threshold up to 100 copies of the target sequences. A small cohort of field grapevine samples, with a known status of infection by mixtures of the target viruses or free of them, respectively, was successfully tested for the evaluation of the amplicons Tm.

Based on the European Union Directive 2005/43-EC, grapevine stock materials (*Vitis vinifera* cultivars as well as American rootstocks) for vegetative propagation must not contain, at any level and as a minimal sanitary requirement, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3). Since tools for diagnosis were needed to assess the sanitary status of plant material, serological tests (mainly ELISA) and nucleic acid-based methods have been developed and applied for their detection (Rowhani et al., 2005; Maliogka et al., 2015). However, ELISA generally achieved low sensitivity on implementing programs of phytosanitary certification whereas high sensitivity and specificity are necessary. While a test based on traditional gel analysis PCR amplicons was developed for the simultaneous detection of different grapevine viruses in a single tube (Gambino and Gribaudo, 2006), this method unfortunately still requires an open-tube manipulation that causes a decreasing throughput and increases the risk of PCR product contamination that can lead to false positive results (Cheng et al., 2013). Multiplex Real-Time PCR does not need post-amplification procedures, therefore allowing a rapid analysis with a larger sample throughput, limiting multiplex PCR drawbacks (Garrido et al., 2012). Real time PCR technologies are based on fluorescent probes or on double stranded (ds) DNA-binding fluorescent dye assays. Generally, the probes are able to quantify single targets in the multiplex RT-PCR, but are expensive and present some false-negative rates for virus detection, caused by sequence variability within the probe-binding site (Papin et al., 2004). Moreover, when several targets are detected, the multiplex RT-PCR with probe dyes may lead to a false positive result due to overlapping dye wavelengths. Indeed López-Fabuel et al. (2013) used a compensation colour assay strategy to properly detect five grapevine viruses in a multiplex TaqMan RT-qPCR. Osman et al. (2013) detected simultaneously grapevine vitiviruses using triplex RT-qPCR, while Bruisson et al. (2017) developed three quadruplex assays (with some sets containing degenerate primers) with an internal control.

Therefore, despite the dsDNA-binding dyes are only able to detect the total amount of accumulated amplicons during the multiplex PCR reaction, they are cheaper than probes (Higuchi et al., 1992) and useful for qualitative assays. In the RT-PCR, when the dsDNA dyes are used, the reaction specificity should be evaluated at the end of reaction using the melting curve analysis (MCA). Generally, the MCA output is a peak specific for the amplicon generated. This is characterized by a specific melting temperature (Tm) influenced by the amplicon length, GC/AT ratio and sequence of the fragment (Wehrle et al., 2010). Therefore, it is possible to distinguish pathogens in qualitative multiplex real time PCR by different peak melting temperatures (Cheng et al., 2013; Khan et al., 2011; Zheng et al., 2013; Rao et al., 2014; Zheng et al., 2016). MCA has

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Table 1
Primers designed for GLRaV-3, ArMV, GFLV, GLRaV-1 multi-RT-PCR and *TIP41-like protein* positive control (CTRL+) used for sing-RT-PCR.

Primer	Sequence (5'-3')	Tm (°C)	multi-RT-PCR primer [μM]	Amplicon size (bp)	uMELT* Tm (°C)
F-GLRaV-3	CCGGAATCTGAGACTCTTACAC	54.6	0.375	92	78
R-GLRaV-3	TCCAAAGCTATTCCCTTGCC	55.2			
F-ArMV	AGTGCCTACAAGAGTGTGTCC	56.7	0.125	196	81
R-ArMV	TAGTGACCCCGTTCCATTCAC	56.7			
F-GFLV	TGGAACGGGACCACTATGG	56.2	0.375	185	83
R-GFLV	GCGATAGGTCCTGCTATTGC	55.7			
F-GLRaV-1	CGGAGTATAACTCGTTCAAGCG	55.4	0.125	221	86,5
R-GLRaV-1	CGAAAATAACACGCTCACGACG	56.6			
F-CTRL+	GAAAGGAAGCGACTGAGAGAG	54.9	Only for sing-RT-PCR	230	
R-CTRL+	TCCGGCAAGTGTGATGTTTG	56.0			

* uMELT Tm represents the amplicon temperature prediction.

Table 2
Amplicon melting temperature (Tm) results obtained with single-RT-PCR (sing) from the analysis of multi-infected field samples and compared with those obtained with multi-RT-PCR (multi). For the single-RT-PCR, the threshold cycle (C_T) is shown for each positive field sample. *Tip41-like protein* gene was used as housekeeping control (CTRL) in single-RT-PCR amplification. nd: not detected.

GRAPEVINE SAMPLES (cultivar)	GLRaV-3			ArMV			GFLV			GLRaV-1			CTRL
	sing		multi	sing		multi	sing		multi	sing		multi	CT
	CT	Tm	Tm	CT	Tm	Tm	CT	Tm	Tm	CT	Tm	Tm	
1. Pinot noir	22.73	77.9	77.7	25.3	79.7	79.7	nd	nd	nd	nd	nd	nd	27.5
2. Prunesta	nd	nd	nd	nd	nd	nd	nd	nd	nd	17.2	84.5	84.8	29.4
3. Dolciolo	30.2	78.4	nd	nd	nd	nd	28.1	82.6	82.8	16.8	85.6	85.7	27.4
4. Maruggio	27.3	78,0	77.6	nd	nd	nd	25.8	83.0	83.8	nd	nd	nd	29.1
5. Uva Attina	22.2	78.4	78.0	nd	nd	nd	21.5	83.2	82.7	nd	nd	nd	27.7
6. Impigno	27.6	78.2	78.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	29.7
7. Trebbiano	23.9	78.2	78.0	nd	nd	nd	22.4	82.1	82.0	26.7	85.3	85.6	29.1
8. Ottavianello	34.6	78.2	78.0	nd	nd	nd	26.4	82.3	82.4	nd	nd	nd	27.7
9. Chianga palmento	23.9	78.5	78.1	nd	nd	nd	22.2	82.9	82.7	nd	nd	nd	29.2
10. Sangiovese	22.6	78.5	78.0	nd	nd	nd	34.0	82.5	82.7	24.9	86.0	86.0	29.9
11. Falanghina	23.0	78.5	78.1	nd	nd	nd	28.9	82.5	82.4	32.8	86.0	86.1	28.8
12. Syrah	24.3	78.5	78,0	17.0	79.6	79.7	26.9	82.5	82.5	nd	nd	nd	28.1
13. Somarello rosso	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	29.1
14. Scannapecora	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	29.4
15. S. Pietro	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	27.7
16. Pedicinaro	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	27.7

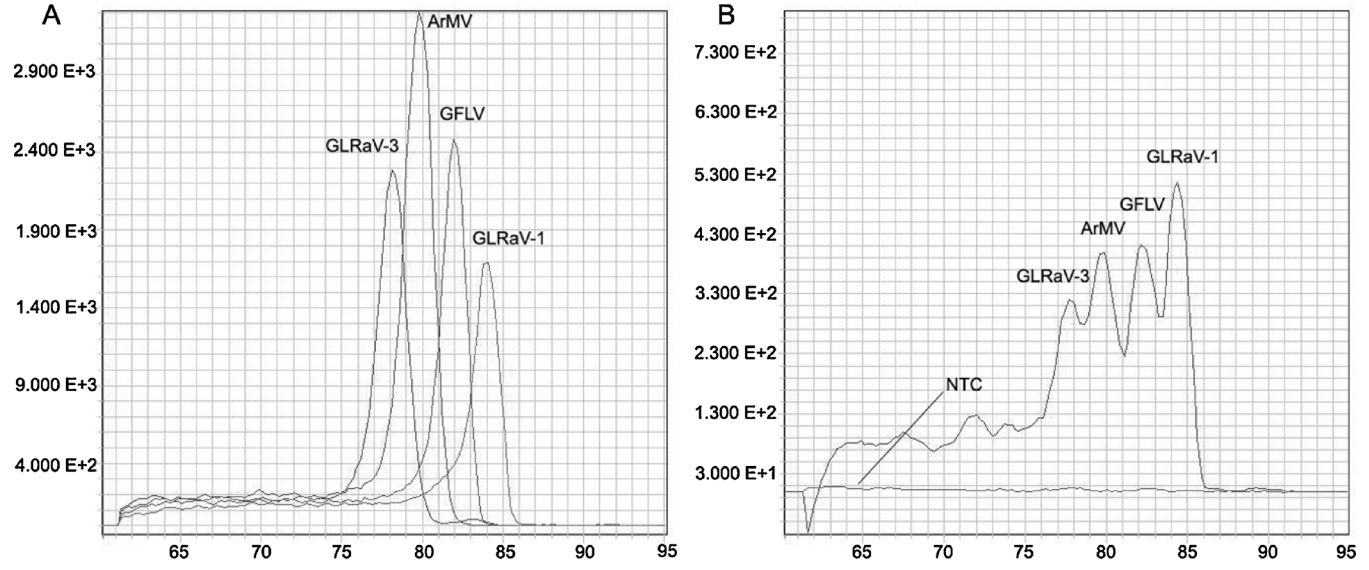

Fig. 1. Specificity of the EvaGreen® multi-RT-PCR protocol for the four grapevine viruses tested, as assessed by MCA. Dissociation curves show Tm (x-axis) plotted against the first derivative of the fluorescence (y-axis). (A) Results obtained for each virus plasmid when tested by the sing-RT-PCR protocol. (B) Each distinct peak corresponded to a specific virus target plasmid amplified by multi-RT-PCR protocol, where the NTC did not show any peak.