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Development of real-time PCR based assays for simultaneous and improved detection of citrus viruses

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Abstract Citrus, one of the most economically important crops, is susceptible to a number of arthropod- and graft-transmissible pathogens. Rapid and reliable methods for detecting multiple pathogens are important for routine diagnosis by reducing time, labour and costs. To this end, primers and TaqMan probes for *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV) detection by singleplex real-time (q) reverse transcription (RT)- PCR were initially designed. Further optimizations included the development of a multiplex (m) RT-qPCR assay to detect simultaneously CPsV, CVV, and *Citrus tristeza virus* (CTV) in a single reaction. When 10-fold serial dilutions prepared using total RNAs from CPsV- and CVV-infected plants were tested, RT-qPCR assays proved to be 100 and 1000 times more sensitive than conventional RT-PCR, respectively. The target viruses were effectively identified by mRT-qPCR in field-infected clementine and sweet orange trees. The optimized multiplex assay proved to be as sensitive as the singleplex tests, thus providing a valuable alternative tool for detection of these citrus viruses.

Keywords *Citrus* viruses · Detection · Real time-PCR · Singleplex assay · Multiplex assay

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Introduction

Citrus, a most important crop in Mediterranean countries and several other areas of the world, is susceptible to several arthropod-transmitted pathogens and numerous other graft-transmissible agents. Among the first group of pathogens, *Citrus Tristeza virus* (CTV), genus *Closterovirus*, family *Closteroviridae*, is the causal agent of various diseases with dramatic effects on citrus crops. Types and severity of symptoms induced by CTV are associated with different strains and scion/rootstock combinations (Roistacher 1991).

Citrus psorosis virus (CPsV) and *Citrus variegation virus* (CVV) are also the causal agents of widespread graft-transmissible diseases (Gonsalves and Garnsey 1974, 1975; da Graça et al. 1991; Martín et al. 2004).

CPsV, the type species of the genus *Ophiovirus*, has a genome consisting of three single-stranded RNAs of negative polarity that have been totally sequenced (Martín et al. 2005), while CVV, a definitive species

of the genus *Ilarvirus*, family *Bromoviridae* has a tripartite, positive-sense single-stranded RNA genome (Scott and Ge 1995; Li et al. 2008).

A number of diagnostic tools has been developed to detect CTV, CPsV and CVV. Indexing on Mexican lime seedlings for CTV, sweet orange seedlings for CPsV, Eureka lemon or citron Etrog for CVV (Wallace and Drake 1951, Roistacher 1991; Roistacher 1993), has been widely used in the past; however the assay is time consuming and relatively expensive for growing and maintaining indicator plants. More recently, polyclonal antisera, specific monoclonal antibodies (MAb) or recombinant antibodies (Garnsey 1974; Bar-Joseph et al. 1979; Cambra et al. 1991, 2000; Garnsey et al. 1993; Nikolaeva et al. 1996; García et al. 1997; Alioto et al., 1999; Potere et al. 1999; D'Onghia et al. 2001; Martín et al. 2002; Loconsole et al. 2006) have been used for rapid detection of CTV, CPsV, CVV by enzyme-linked immunosorbent assay (ELISA) or direct tissue blot immunoassay (DTBIA). However, the use of serological tests may be limited by the low titre of the target viruses in certain periods of the year, by the uneven distribution of the viruses in infected plants and, particularly for CVV, by the short season in which the young flushes are available for testing (Davino et al. 1988; Roistacher 1993; Mathews et al. 1997; Loconsole et al. 2009). To overcome these limitations and improve the sensitivity and detection limits of the assays, PCR-based and molecular hybridization techniques have been developed in the last ten years for CTV, CPsV and CVV detection (Hilf and Garnsey 2000; Bennani et al. 2002; Martín et al. 2004; Roy et al. 2005; Barbarossa and Savino 2006; Rosa et al. 2007; Loconsole et al. 2009). As an alternative to traditional PCR, several real-time (q) RT-PCR protocols have been successfully used to detect and differentiate CTV strains (Ruiz-Ruiz et al. 2007, 2009; Bertolini et al. 2008; Saponari et al. 2008; Yokomi et al. 2010).

A valuable feature of molecular techniques is the possibility to detect several pathogens in a single reaction, a very attractive possibility for citrus which are often infected by two or more agents. Several strategies have been successfully employed for the simultaneous detection and identification of several viruses in a single PCR by using either multiple primer pairs (multiplex RT-PCR) or a polyvalent primer pairs (polyvalent PCR) (James 1999; Bertolini et al. 2001; Ito et al. 2002; Foissac et al. 2001; Roy et al. 2005; Sanchez-Navarro et al. 2005; Gambino and Gribaudo 2006; Wang et al. 2009). Real-time PCR is known to be more sensitive, faster and relatively easier to perform compared with conventional PCR (reduced cycle times and steps, no need for electrophoresis, staining and gel documentation, reduced risk of contamination). Although, several RT-qPCR protocols are available for CTV, as yet, no real-time reagents specific for CPsV and CVV have apparently been developed. Attempts were therefore made in this work to develop a reliable test to detect CPsV and CVV by RT-qPCR, and subsequently develop a multiplex (m) one-step RT-qPCR assay to detect CTV, CPsV and CVV in a single reaction.

Material and methods

Virus sources

CPsV and CVV isolates were from the collection of citrus pathogens belonging to the University of Bari (Table 1). Virus isolates were maintained in sweet orange plants grafted on sour orange and originated from southern Italy, Spain, Lebanon and the USA. CPsV isolate Ps101, CVV isolate CVV300 and CTV isolate IT08 were used to develop and optimize the mRT-qPCR assays.

To validate both assays, 230 field samples (182 sweet orange and 48 clementine trees) were collected between September and December 2008, from 15–30 year-old trees of sweet orange and clementine grafted on sour orange. Surveyed plots were located in a CTV-contaminated area of Apulia (southern Italy). Samples consisted of 4 young shoots with leaves collected around the canopy, from symptomless plants as well as from plants showing symptoms of decline, stunting and yellowing.

Isolation of total RNA

Total RNAs (TNAs) from citrus tissues were extracted from 0.2 g of leaf petioles after homogenization with the Mixer Mill300 (Qiagen, Germany), using the procedure described by Foissac et al. (2001). TNAs were then eluted in 150 μl of RNase free water and their concentration was determined using a UV-vis Spectrophotometer.

Table 1 Isolates of citrus viruses and viroid maintained in the collection of the University of Bari and used to validate the singleplex RT-qPCR assays[a]

Isolate	Origin	Field symptoms	Symptoms on indicator plants[b]	Real time RT- PCR C_t values X±SD[c]		
				CPVp	CVVp2	CP25-FAM
Citrus psorosis virus						
Ps101	Southern Italy	OLP, LS, BS	LS, OLP, S	17.35±0.23	0	0
204-X		OLP, LS	OLP, LS	21.75±0.25	0	0
207-X		LS, BS, S	RS, LS	16.95±0.39	0	0
321-X		RS	LS, S	17.86±0.28	0	0
365-X		OLP	LS	18.33±0.19	0	0
391-X		LS	LS	20.33±0.16	0	0
77V		LS, OLP	LS, OLP	18.81±0.14	0	0
243-X	USA	BS	LS, S	18.43±0.11	0	0
245-X		LS	LS, S	19.08±0.27	0	0
248-X		LS	S	16.73±0.22	0	0
250-X		RS	LS, RS, S	21.05±0.17	0	0
304-X	Spain	RS	LS, RS	18.24±0.37	0	0
316-X		LS	LS, OLP	18.72±0.19	0	0
317-X		BS, LS	LS, RS	19.83±0.23	0	0
9N36 (2)	Lebanon	OLP	LS	19.89±0.17	0	0
9N56(1)		OLP	LS	17.69±0.16	0	0
Citrus variegation virus						
CVV300	Southern Italy	CL, C, V	CL, C, LD	0	13.44±0.17	0
60-X		LD, C	CL, C	0	14.25±0.19	0
209-X		asymptomatic	CL, C, LD	0	14.07±0.10	0
314-X		C	CL, C, LD	0	13.92±0.15	0
327-X	USA	CL,C	CL, C, LD	0	14.69±0.12	0
Citrus tristeza virus						
IT-02				0	0	14.92±0.13
IT-06				0	0	14.25±0.23
IT-08				0	0	15.31±0.18
Citrus leaf blotch virus						
15K	Southern Italy	asymptomatic	Not done	0	0	0
Citrus exocortis viroid						
VdX	Southern Italy	Not known	Not done	0	0	0

BS bark scaling; *LS* chlorotic flecking or spotting in young leaves; *OLP* oak leaf pattern; *RS* ringspot in leaves; *S* shock reaction (leaf shedding and necrosis of young shoots); *LV* variegation in young leaves; *CL* crinkly leaf; *C* chlorotic leaves; *V* variegation on the young leaves; *LD* leaves deformation.

[a] Isolates in bold were used to optimize the qRT-PCR protocols and test the primer/probe sets.

[b] Dweet tangor and Sweet Orange cv Madam Vinous (MV) were used as indicator plants for CPsV, Eureka lemon for CVV; Mexican lime, MV and sour orange for CTV.

[c] X: Average of two wells per real time assay; SD Standard deviation.

TaqMan probes and primers design

Several primers and TaqMan probes specific for CPsV and CVV were designed within the coat protein (CP) region of both viruses using Primer Express software (Applied Biosystems, USA) (Table 2). Multiple alignments of the CP gene from various CPsV and CVV isolates were made using ClustalW

Table 2 Sequence of forward and reverse primers and TaqMan probes used for real-time reverse transcription PCR for *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV). Best performing primers and probes are in bold

Primer/probe	Sequence 5′-3′	Expected amplicon size (bp)	Primer/probe location
CPsV			
CPV200f (forward)	**GCWGGWAATCGRTCTGTGAGRTAT**	**106** or 70	**200–223**[a]
CPV237f (forward)	GCAGYTTYCARACHAARCAAAA		237–258[a]
CPV287r (reverse)	**AGCAAWGGCATCARGGAYTC**		**287–306**[a]
CPVp	**Cy5 [b] - TCYCCTGCTGTTGGWGCAACTYC-BHQ1**		**263–285**[a]
CVV			
CVV27f (forward)	GCATGAAGTTTCTGGGTAYAGTTTCC	107	27–52[c]
CVV109r (reverse)	TGGTATCATGGAGCGTTCKTATTTT		109–133[c]
CVVp1	TR[d] -CATTGCYTACATGACCCTACGTTCGGA**-BHQ1**		75–101[c]
CVV178f (forward)	**GCTCAAGGACTGGCGAAYGT**	**61**	**178–197**[e]
CVV219r (reverse)	**GCATGGAACCGACCAACAAC**		**219–238**[e]
CVVp2	**TET[f]-CGACGRTCACAACCACAA-MGB**		**199–216**[e]

[a] Primer location based on EMBL accession number AM409317.

[b] Probe labeled with the reporter Cy5 (Cyanine 5).

[c] Primer location based on EMBL accession number AJ508381.

[d] Probe labeled with the reporter Texas Red.

[e] Primer location based on GenBank accession number EU650678.

[f] Minor groove binding (MGB) probe labeled with the reporter TET (tetrachloro-6-carboxyflurescein).

(Thompson et al. 1994) and GeneDoc (Version 2, 1991). Degenerate nucleotides were included in the primers and probes to ensure broad spectrum reactivity. The CPsV-specific TaqMan probe (CPVp) was labelled at the 5′ with Cyanine 5 (CY5), whereas the CVV-specific TaqMan probes (CVVp1 and CVVp2) were labeled with Texas Red or TET.

For CTV detection, the primers CP25f/r and the FAM- (6-carboxyfluorescein) labelled CP25-probe (Saponari et al. 2008) were used.

Real-time one step RT-PCR assays

Singleplex and multiplex RT-qPCR reactions were performed in a final volume of 25 µl on the CFX96™ Real time System (Biorad).

Singleplex reactions for CPsV and CVV were set up in 1X iScript one-step RT-PCR Kit for probes (Biorad, USA), and contained 320nM of each primer and 160nM of the specific TaqMan probe. The amplification profile was one cycle at 50°C for 5 min and then 10 min at 95°C followed by 40 cycles at 95°C for 20 s and 55/58/60/ °C for 40 s. The best combination of primers/TaqMan probes and the optimal annealing temperature were selected as the one that gave the highest end reporter fluorescence (RFUs) and the lowest threshold cycle (C_t).

Triplex (CPsV, CVV and CTV) assay were performed in 1X IQ-Multiplex power mix (Biorad) additioned with 15U of Multiscribe-RT (Applied Biosystem); primers concentration varied from 160 to 320 nM, whereas probes were concentrated from 80 to 160 nM per reaction. The primers/probes concentrations were optimized to reduce the competition among virus templates, thus obtaining the lowest ΔCt between singleplex and multiplex assays.

The cycling profile of the mRT-qPCR consisted of 5 min incubation at 50°C, then 10 min incubation at 95°C followed by 40 cycles at 95°C for 20 s and 58/60/62°C for 40 s.

The efficiency and sensitivity of singleplex and multiplex RT-qPCR assays were determined using six 10-fold (from 1 to 10^{-5}) serial dilutions prepared using a starting amount of 25 ng of total RNAs from isolates Ps101, CVV300 and IT-08 diluted in TNAs from healthy Madame Vinous. All standard curves were generated using two replicates of each dilution in two independent assays. Conventional RT-PCR reactions

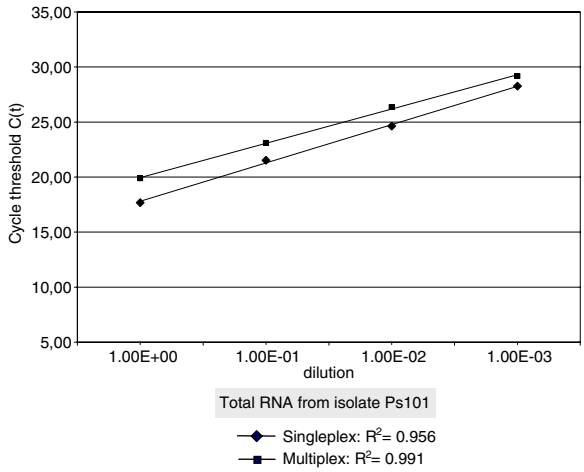

Fig. 1 Standard curves and linear regression (R^2) obtained in singleplex and multiplex RT-qPCR with primers/probes for *Citrus psorosis virus*, using 10-fold serial dilution (from 1 to 10^{-3}) of total RNA extracted from Ps101 isolate

were performed using the same dilution series following the procedures described for CPsV by Barthe et al. (1998) and for CVV by Loconsole et al. (2009).

When singleplex and multiplex RT-qPCR assays were used for field tests, *ca.* 25 ng of plant TNAs were always used in each reaction. All assays included positive, negative and non-template controls (NTC).

Results

Development of singleplex RT-qPCR assay

The newly designed primers and TaqMan probes successfully detected CPsV and CVV in Ps101 and CVV300 sources, respectively (Table 1). However, the best results were obtained with the primers/probes shown in Table 2 using the following RT-qPCR conditions: (i) for CPsV, primers CPV200f/CPV287r at 320 nM with CPVp probe at 160 nM; (ii) for CVV, primers CVV178f/CVV219r at 320 nM with CVVp2 probe at 160 nM. For both templates the optimal annealing temperature was 58°C.

Both selected primers/probe sets were able to correctly identify the isolates from the collection (Table 1). No amplification was obtained with healthy controls or with plant tissue infected with other citrus viruses and viroid (Table 1).

The qPCR efficiencies (E), determined using the 10-fold serial dilutions of total RNA from PS101 and CVV300, were respectively 110% for CPsV with a regression coefficient (R^2) of the standard curve of 0.991 (Fig. 1) and 115% for CVV with a regression coefficient (R^2) of the standard curve of 0.993 (Fig. 2).

Both RT-qPCR assays were able to detect CPsV and CVV up to a dilution of 10^{-3} and 10^{-4}, respectively; whereas gel electrophoresis of the conventional RT-PCR products showed a clear signal of amplification only up to dilution of 10^{-1} (data not shown).

Multiplex RT-qPCR assay

The optimized multiplex RT-qPCR conditions included the annealing temperature of 60°C and the following primers/probes concentration: (i) for CVV, 160 nM of CVV178f/CVV219r and 80 nM of CVVp2; (ii) for CPsV, 320 nM of CPV200f/CPV287r and 160 nM of CPVp; (iii) for CTV, 160 nM of P25f and P25-FAM, and 320nM of P25r.

No Ct changes were observed when each single virus template was detected using the reaction mix containing all three primers/probe sets (data not shown), indicating that no interference occurred among the primers/probe sets.

When the six 10-fold dilutions prepared with the reference isolates IT-08, Ps101 and CVV300 were artificially mixed (1:1:1 volume) and used as a template for the multiplex assay, all targets were clearly detected by the specific probes up to a dilution of 10^{-4} (CVV) and 10^{-3} (CTV and CPsV) (Figs. 1, 2 and 3).

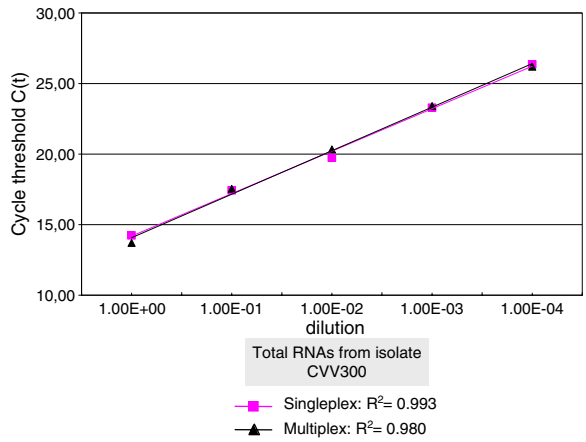

Fig. 2 Standard curves and linear regression (R^2) obtained in singleplex and multiplex RT-qPCR with primers/probes for *Citrus variegation virus*, using 10-fold serial dilution (from 1 to 10^{-4}) of total RNA extracted from CVV300 isolate

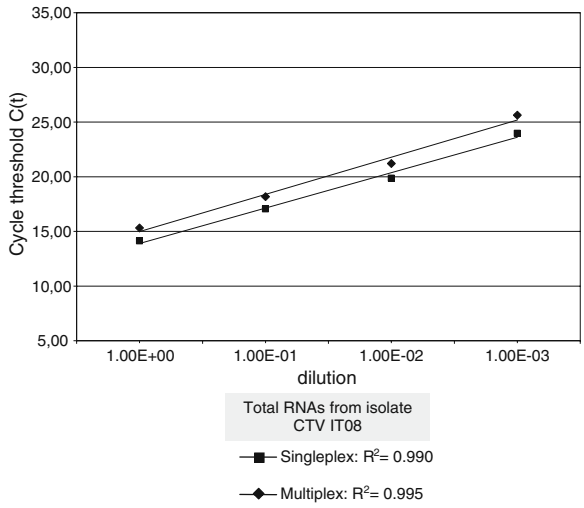

Fig. 3 Standard curves and linear regression (R^2) obtained in singleplex and multiplex RT-qPCR with primers/probes for *Citrus tristeza virus*, using 10-fold serial dilution (from 1 to 10^{-4}) of total RNA extracted from CTV IT-08 isolate

Thus, for all target viruses the sensitivity achieved in multiplex assays was comparable to that obtained in the singleplex assay. For each dilution series the overall differences in Ct values, between multiplex and singleplex assay, were +0.03, +1.61, +1,33 for CVV, CPsV and CTV respectively. Similarly to the singleplex, qPCR efficiencies obtained for each target virus in multiplex were 110%, 101% and 86% for CPsV, CVV and CTV, respectively.

No signal was detected in the healthy controls or in the NTC wells.

Field validation

CTV and CPsV in single or multiple infections were detected in a total of 111 of the 230 field samples (182 samples of sweet orange and 48 of clementine), whereas none of the tested samples resulted infected by CVV. Forty-four sweet orange and 4 clementine samples resulted dually infected by CTV and CPsV. Single infections by CTV were recorded in 17 sweet orange and in 10 clementine samples. While single infections caused by CPsV were only found in 36 samples of sweet orange. Singleplex and multiplex RT-qPCR results showed 100% of agreement.

Table 3 reports the Ct values obtained for the subset of 24 samples analyzed. Ct-shift values between the multiplex and the singleplex assays ranged for CTV from +0.07 to +1.68, for CPsV from +0.01 to +2.31.

Discussion

Real-time PCR is being used as a powerful tool for the rapid and sensitive detection and strain differentiation of woody plant pathogens. A valuable advantage is in the multiplexing capability, which allows for high throughput pathogens screening. To date, multiplex RT-qPCR has been successfully used to detect woody plant-infecting viruses and to differentiate CTV and PPV strains (Varga and James 2006; Pallas et al., 2008; Ruiz-Ruiz et al. 2009; Ananthakrishnan et al. 2010; Yokomi et al. 2010), whereas, none of these protocols were aimed at identifying multiple viruses infections. To our knowledge, this is the first one step multiplex RT-qPCR successfully developed to detect three of the most common citrus viruses.

CPsV and CVV primers/TaqMan probes as designed here, effectively detected all geographically and biologically different CPsV and CVV isolates (Table 1).

Standard curves generated in singleplex assays with the newly developed CVV and CPsV primers/probe sets, had correlation coefficients (R^2) of 0.993 and, 0.991 respectively, indicating high reproducibility and amplification efficiency in the optimal range of 90–120%. When these primers/probe sets were multiplexed with the CTV specific P25 primers/probe set, the R^2 values of the standard curves generated using the same 10-fold dilutions ranged from 0.980 for the CVV to 0.991 for the CPsV and 0.995 for CTV, thus indicating high reproducibility, no reduction in the amplification efficiency, optimal standardization of the assays, no interference or competition among primers/probes sets when they were mixed. When compared to conventional RT-PCR, the CPsV and CVV singleplex and multiplex assays were 10^2 and 10^3 times more sensitive, respectively.

No significant variations in Ct values were observed when the detection of each single virus was performed in singleplex or multiplex reactions.

When both assays were validated in the field, no trees infected with CVV were found probably due to the low incidence (3%) of this virus in the monitored area (Fatone et al. 2003) and because no lemon trees, the preferential CVV host, were included in the trees sampled. The lower incidence

Table 3 Cycle threshold (Ct) values obtained for *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV) and *Citrus variegation virus* (CVV) in multiplex (Mx) and singleplex (Sx) assays using three reference isolates and a subset of 24 sweet orange and clementine field trees. Clementine samples are shadowed

ID Citrus Plants	CTV		CPsV		CVV	
	Mx Ct values X±SD[a]	Sx Ct values X±SD[a]	Mx Ct values X±SD[a]	Sx Ct values X±SD[a]	Mx Ct values X±SD[a]	Sx Ct values X±SD[a]
Reference isolates						
IT08	13.87±0.03	13.69±0.05	0	0	0	0
Ps101	0	0	17.70±0.01	16.87±0.03	0	0
CVV300	0	0	0	0	12.88±0.48	12.73±0.25
Field samples						
P.8	14.35±0.02	13.36±0.07	24.33±0.08	24.89±0.12	0	0
P.19	19.60±0.01	18.89±0.02	21.93±0.07	21.80±0.03	0	0
P.21	18.48±0.10	18.19±0.09	21. 13±0.11	20.84±0.05	0	0
P.23	19.51±0.11	19.43±0.05	0	0	0	0
P.24	0	0	20.04±0.07	17.93±0.13	0	0
P.25	14.83±0.03	14.09±0.13	20.35±0.17	19.03±0.20	0	0
P.26	16.84±0.07	16.51±0.11	18.11±0.06	17.22±0.14	0	0
P.53	0	0	19.4 5±0.02	17.32±0.11	0	0
P.59	13.97±0.08	13.28±0.10	18.44±0.04	17.29±0.17	0	0
P.62	13.30±0.11	11.90±0.03	0	0	0	0
P.73	16.05±0.08	15.54±0.11	20.04±0.17	19.21±0.20	0	0
P.86	0	0	19.00±0.22	21.88±0.18	0	0
P.88	14.62±0.12	13.05±0.07	19.40±0.13	18.25±0.16	0	0
P.94	15.54±0.11	14.35±0.03	0	0	0	0
P.95	11.79±0.02	11.20±0.05	0	0	0	0
P.97	16.65±0.10	16.91±0.04	19.08±018	17.47±0.21	0	0
P.103	0	0	22.02±0.17	20.47±0.08	0	0
P.108	0	0	19.98±0.23	17.67±0.15	0	0
P.109	17.56±0.02	16.91±0.07	21.35±0.16	21.14±0.09	0	0
P.113	19.01±0.03	17.81±0.09	24.07±0.07	22.57±0.01	0	0
P.115	14.89±0.15	13.52±0.07	0	0	0	0
P. 116	13.62±0.03	12.15±0.08	25.61±0.03	24.12±0.08	0	0
P.117	14.11±0.12	14.04±0.05	20.40±0.10	18.92±0.22	0	0
P.118	14.99±0.07	14.79±0.02	0	0	0	0

[a] X: Average of two wells per real time assay; *SD* Standard deviation.

of CPsV detected in clementine (8.3%) than in sweet orange (43.9%) is in agreement with previous data (Roistacher 1991). For CTV, the incidence assessed by mRT-qPCR confirmed the data from the CTV surveys annually carried out in the same area (A.M. D'Onghia, personal communication). Interestingly, a high correlation was found between the presence of CTV and the occurrence of decline and yellowing symptoms on sweet orange trees, whereas, CTV-infected clementine trees did not show any specific symptomatology.

In conclusion, a one step real-time RT-PCR protocol has been successfully developed and validated to detect CVV and CPsV in citrus trees. Rapid and accurate multiple detection of CTV, CPsV and CVV was achieved using the triplex one step real-time RT-PCR protocol. These assays may prove useful as a tool to support quarantine, eradication

and certification programs, resulting faster, more sensitive, relatively easier to perform and less cost effective than the conventional detection procedures.

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