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Rapid identification of *Trialeurodes vaporariorum*, *Bemisia tabaci* (MEAM1 and MED) and tomato-infecting criniviruses in whiteflies and in tomato leaves by real-time reverse transcription-PCR assay

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Abstract

The whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* (Hemiptera Aleyrodidae) are harmful pests of vegetable and ornamental crops in many countries. Also, they are vectors of emergent viruses on tomato including the criniviruses (Closteroviridae genus *Crinivirus*) Tomato chlorosis virus (ToCV) and Tomato infectious chlorosis virus (TICV). Since different vectors are involved in the transmission of both viruses (ToCV is transmitted by *B. tabaci*, *Trialeurodes abutiloneus* and *T. vaporariorum* while TICV is transmitted only by *T. vaporariorum*), and they induce similar symptoms on tomato plants, a sensitive and specific diagnosis method is desirable. In addition, a rapid discriminating method of the vectors is essential for monitoring and control activities and epidemiological studies. For these reasons, a combined protocol based on one-step multiplex real-time reverse transcription (RT)-PCR has been developed for the identification of *T. vaporariorum*, two invasive species of the complex *B. tabaci* (MEAM1 and MED) and for the specific detection of ToCV and TICV in whiteflies and plants.

Key words: Aleyrodidae, virus vector, tomato yellowing, molecular diagnosis.

Introduction

During the last decades, whitefly populations (Hemiptera Aleyrodidae) have rapidly increased throughout the world as well as the associated virus diseases (Wisler *et al.*, 1998b; Brown, 2007; Wintermantel, 2010; Navas-Castillo *et al.*, 2011). Although a high number of whitefly species have been described, only a few are virus vectors and these include *Bemisia tabaci* (Gennadius) and *Trialeurodes vaporariorum* (Westwood). *B. tabaci* is considered the most important pest due to its wide distribution, host range and capacity to transmit most of the whitefly transmitted-viruses. The viruses transmitted by *B. tabaci* include members of the genera *Begomovirus* (Geminiviridae), *Crinivirus* (Closteroviridae), *Carlavirus* (Betaflexiviridae), *Ipomovirus* (Potyviridae) and *Torradovirus* (Secoviridae) (Jones, 2003; Navas-Castillo *et al.*, 2011; EFSA, 2013).

B. tabaci includes a complex mix of genetically but not morphologically distinguishable populations, which have been referred as biotypes. Recently, it has been proposed that *B. tabaci* is a complex of different species (Dinsdale *et al.*, 2010; De Barro *et al.*, 2011). Different molecular tools have been developed in the last decade to study this genetic diversity and to identify the different biotypes/species (Guirao *et al.*, 1997; Frohlich *et al.*, 1999; Cervera *et al.*, 2000; De Barro *et al.*, 2000). Middle East-Asia Minor 1 (MEAM1, formerly biotype B) (Demichelis *et al.*, 2000) and Mediterranean (MED, formerly biotype Q) are the most common and polyphagous species of the *B. tabaci* complex found in Italy (Demichelis *et al.*, 2000; Bosco *et al.*, 2001); they both are responsible for the transmission and emergence of begomoviruses and some criniviruses worldwide. *T. vaporariorum* is also a polyphagous pest but transmits a limited number of viruses, all within the genera *Crinivirus* and *Torradovirus* (Wisler *et al.*, 1998a; Jones, 2003; Brown *et al.*, 2007; Navas-Castillo *et al.*, 2011). Compared to *B. tabaci*, fewer studies were carried out with *T. vaporariorum*. Recent studies analyzed the genetic variation of this whitefly using COI gene and the internal transcribe spacer (ITS) sequence of ribosomal DNA, finding a very low genetic diversity in different populations of *T. vaporariorum* (Roopa *et al.*, 2012; Prijović *et al.*, 2014). Other authors, using biochemical and allozime analysis, found two distinct populations of *T. vaporariorum* separated by geographical barriers (Shin *et al.*, 2013).

The genus *Crinivirus* includes a number of species emerged in the past two decades (Wintermantel and Hladky, 2010). The criniviruses *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* (ToCV), firstly identified on tomato (*Solanum lycopersicum* L.) in USA and characterized in the mid of 1990 (Duffus *et al.*, 1996; Wisler *et al.*, 1998b), are now emergent viruses worldwide, becoming a serious threat in many countries including those in the Mediterranean Basin (Tzanetakis *et al.*, 2013). In Italy, both criniviruses have been found in several regions on protected tomato (Accotto *et al.*, 2001; Vaira *et al.*, 2002; Davino *et al.*, 2007) and other crops (Parrella, 2008).

TICV is transmitted exclusively by *T. vaporariorum* (Duffus *et al.*, 1996), while ToCV is transmitted by *Trialeurodes abutiloneus* (Haldeman), *T. vaporariorum* and the New World 1 (NW1, formerly biotype A), MEAM1 and MED species of the *B. tabaci* complex (Wisler *et al.*, 1998a). Studies have shown differences in the efficiency to transmit ToCV among species, showing that *T. abuti-*

loneus and *B. tabaci* MEAM1 and MED are more efficient than *B. tabaci* NW1 and *T. vaporariorum* (Wintermantel and Wisler, 2006). Both viruses are transmitted in a semi-persistent manner to plant hosts. In addition to tomato, the viruses infect several cultivated plants, ornamental and weeds that represent a potential virus reservoir and a source for whitefly feeding and colonization resulting in movement of the virus into the near fields (Wintermantel, 2010).

A number of studies on epidemiology, virus-vector species specificity and diagnosis were made during the last years. Sensitive molecular techniques were developed to identify TICV and ToCV in plants and insects, the most recent by real-time RT-PCR (Morris *et al.*, 2006; Papayiannis *et al.*, 2011; Cavalieri *et al.*, 2011; Tiberini *et al.*, 2011). Concerning vectors, real-time PCR was also developed for *B. tabaci* identification (Zhang *et al.*, 2007) and to differentiate the MEAM1 and MED species (Jones *et al.*, 2008; Papayiannis *et al.*, 2009). Since a different host preference is usually shown by different parasitoids, the correct identification of these whitefly species is fundamental for applying an effective biological control strategy of the vectors which has a basic role in reducing their attacks to crops, within an integrated pest management strategy of primary importance especially in lack of commercial tomato varieties resistant to the two viruses.

The present paper describes combined protocols based on one-step real time RT-PCR (Heid *et al*., 1996; Mackay *et al*., 2002; Bustin *et al*., 2005) that were specially performed to deliver a complete and rapid method to investigate the distribution and prevalence of *T. vaporariorum* and *B. tabaci* in the two main Italian islands, Sardinia and Sicily. Also, the real-time RT-PCR assay was developed for rapid identification of vector species and simultaneously detection of tomato criniviruses into their bodies. Because vector species identification is not an easy task based on whitefly morphology, specific primers and TaqMan probes were designed to allow identification in a single reaction of MEAM1 and MED species of *B. tabaci* as well as of *T. vaporariorum*.

Materials and methods

Whitefly and plant sampling in tomato greenhouses

Whitefly adults were collected during 2011 in four Sicilian and one Sardinian tomato greenhouses (table 1) by means of a manual aspirator and maintained in 70% ethanol at 4 °C until use. At the same time, symptomatic leaves colonized by the captured whiteflies were also collected and analyzed to confirm the presence of TICV or ToCV infection. *T. vaporariorum*, *B. tabaci* MEAM1 and *B. tabaci* MED populations were also identified from different localities to be used as controls in real-time TaqMan assays. TICV and ToCV positive controls from CRA-PAV collection, validated in a test performance study (ARNADIA Italian project) (Manglli *et al.*, 2013), were used in all assays.

Identification of whitefly species

Each pooled sample of whitefly individuals were discriminated exclusively on a morphological basis (Martin *et al.*, 2000; EPPO, 2004), through both initial field observations and subsequent lab analysis by means of a stereomicroscope.

For *B. tabaci* species discrimination, a molecular technique was applied based on microsatellite marker BEM 23 described by De Barro *et al.* (2003) and used in surveys in Serbia (Žanić *et al*., 2005), Sardinia (Ortu *et al.*, 2007), Sicily (Cavalieri and Rapisarda, 2008), and Tunisia (Bel-Kadhi *et al.*, 2008). Therefore, five specimens from each *B. tabaci* population (samples and controls) were individually subjected to DNA extraction according to the protocol described by Walsh *et al.* (1991) and modified by De Barro and Driver (1997). The PCR reaction was conducted using the primers forward BEM 23 (5'-CGGAGCTTGCGCCTTAGTC-3') and reverse BEM 23 (5'-CGGCTTTATCATAGCTCTCGT-3') (De Barro *et al.*, 2003). All PCR reactions were performed in 20 µl volumes with 1X PCR buffer, 3.5 mM of $MgCl_2$, 125 µM of dNTPs, 7 pmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 µl of DNA template. The cycling conditions were: 94 °C for 5 min, then 40 cycles at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, followed by final cycle at 72 °C for 7 min. Reactions and cycling conditions were conducted in an automated thermal cycler [GeneAmp® PCR System 2700 (Applied Biosystems)]. PCR products were run in 1.6% agarose gel and visualized with SYBR® Safe DNA gel stain (Invitrogen). MEAM1 shows a characteristic band of about 200 bp, while MED shows a band of about 400 bp.

Primer and probe design

In order to achieve the combined identification expected through the present work, a region of the cytoch-

Table 1. Whiteflies collected and used for the one step real time RT-PCR protocol development.

Samples	Locality	Plants	Crop	Date
1	Vittoria (Sicily)	tomato	greenhouse	19/04/2011
2	Vittoria (Sicily)	tomato	greenhouse	01/06/2011
3	Acate (Sicily)	tomato	greenhouse	02/06/2011
4	Acate (Sicily)	tomato	greenhouse	03/06/2011
5	Arborea (Sardinia)	tomato	greenhouse	06/05/2011
T. vaporarioursum	Ispica (Sicily)	zucchini	greenhouse	21/05/2011
B. tabaci MEAM1	Naro (Sicily)	melon	tunnel	25/06/2011
B. tabaci MED	Rome (Lazio)	*Aster* sp.	greenhouse	03/08/2011

Table 2. Probes and primers designed and used in multiplex TaqMan RT-PCR assay.

Name	Probe sequences	Forward primer sequences	Reverse primer sequences
Whiteflies identification (mix 1)			
BEM COI MEAM1	HEX-TTATTTACTATAGGTGGGTTAACTG-BHQ1	5'-TGGCCTTTGATTTACAGGATTTTT-3'	5'-ACACATCTACAGAAGAATTACCAAGAA-3'
BEM COI MED	Cy5-AAGCTTGGTGTAAGCAGA-BHQ2	5'-GAATCTTTTCTTCTTTTGCGTTTAGTAA-3'	5'-AAGGGCTGGTTTATTAATTTTCCA-3'
TRIAL COI	6FAM-TGCAGACTGTTACATTGT-BHQ1	5'-GATGCCTCGACGTTATGTTGATT-3'	5'-CTAACAAGTCTCCCAATAGAAGAAACC-3'
Virus identification* (mix 2)			
TICV	FAM-CGTCAGGTCACCCAAACGCTCTAAGG-BHQ1	5’-GCGGGACATTTTTTATCATATGC-3	5’-TCAGCCCAACATCTTGTAGTTGTT-3’
ToCV	HEX-ACCCCGATGACGGATAAGATTTTCGC-BHQ1	5’-ATCCTTTTGCAGGCGAATAATC-3’	5’-GCCTGACACATAGACATGTAAAAAC-3’
Whitefly 18S	Cy5-CAGCACTTCGCGTGCACACTGGA-BHQ2	5’-CGGGTCCGCGGTTTCT-3	5’-GATCGGCCGGAGTTATTCG-3

* probes and primer designed by Tiberini *et al*. (2011).

rome oxidase sub unit I gene (COI) was selected for discrimination of *T. vaporariorum* and *B. tabaci* MEAM1 and MED. This marker has been employed successfully in the study of genetic variability of whiteflies and *B. tabaci* in particular (Frohlich *et al*., 1999; Zhang *et al*., 2005; Bosco *et al*., 2006; De la Rùa *et al*., 2006; Boykin *et al*., 2007; Dinsdale *et al*., 2010; Roopa *et al*., 2012). A number of sequences of COI region of *T. vaporariorum* and both MEAM1and MED species of *B. tabaci* deposited in GenBank (GenBank accession: gb|AF110708.2|, gb|JF512474.1|, gb|JF693935.1|, emb|AM179445.1|, gb|JF693931.1|, gb|HQ992961.1|, gb|EU760751.1|, gb|EU760747.1|, gb|EU760746.1|, gb|EU760736.1|, gb|JF754925.1|, gb|HQ992959.1|, gb|FJ188589.1|, emb|FN821808.1|, emb|FN821807.1|, gb|JF754925.1|, gi|308026346|) were aligned with Clustal W2 (Larkin *et al*., 2007) to search for variable regions of COI gene between whitefly species, in order to design species-specific TaqMan-probes and primers for every whitefly type.

A total of 10 probes and 17 primers were designed for TaqMan assay using the Primer Express™ version 3.0 software (Applied Biosystems, Life Technologies Corporation, Grand Island, USA) with an amplified fragment size of 67-84 bp. Primers were supplied by Invitrogen Custom DNA Oligos (Life Technologies Corporation) and probes each labeled by differently colored fluorophores were supplied by Sigma-Aldrich (St Louis, USA) and Bio-Fab Research (Roma, Italy). The sequences of probes and primers selected in this work are given in table 2, where internal vector control, designed in the consensus region of rRNA 18s, is also included. Furthermore, primers and probes have been designed in order to use them under the same reaction conditions of TICV and ToCV primers and probes previously designed and validated for TICV, ToCV and tomato endogenous control (cytocrome oxidase gene) (Tiberini *et al*., 2011; Manglli *et al*., 2013; EPPO, 2013).

RNA extraction

Total RNA of 113 whitefly specimens (95 collected in Sicily and 18 collected in Sardinia) and 12 specimens belonging to control whitefly populations (4 for *T. vaporariorum*, 4 for *B. tabaci* MEAM1 and 4 for *B. tabaci* MED) was extracted from single insects using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Before, each specimen was washed with water, dried and homogenized in sterilized tube with pestle. For each sample representing leaves colonized by whiteflies, 1g of tissue was grinded in 5 mL of 0.1M PBS (3.63 g Na_2HPO_4 12 H_2O, 0.24 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl) and 100 µl of the extract were used for total RNA extraction using the above mentioned Qiagen kit.

Triplex real time RT-PCR assays setup for whitefly and virus identification

Triplex TaqMan® RT-PCR assay was set-up in 96-well reaction plates using TaqMan® One Step PCR Master Mix Reagents Kit (Applied Biosystems) as following: 12.5 µl 2X Master Mix, 0.625 µl 40X MultiScibe™ and RNase Inhibitor Mix, 0.75 µl of each primer (10 µM), 0.5 µl of each probe (5 µM) and 1 µl of RNA in a final volume of 25 µl. One reaction mix was prepared for discrimination of the three whiteflies; another mix was prepared for detection of the two viruses into the vectors (table 2). Therefore, every sample was analyzed in the same multi-well preloaded with the two reaction mixes each in one half of the plate. The amplification reaction was carried out as follows: 48 °C for 30 min, 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and an elongation step at 60 °C for 1 min. All reactions were performed in duplicate and the results were visualized and analyzed using SDS software. Probes and primers were tested using RNA from known specimens of the investigated whitefly species, and reference virus isolates. These samples were also used as positive controls. RNA from healthy tomato leaves and virus-free whiteflies were used as negative control Assays were performed in ABIPRISM 7500 Fast. Thresholds cycles (Ct) were automatically calculated and data analyzed by the system software in the thermocycler.

Results

Three primer and probe sets, one for each whitefly type, were selected for multiplex real time RT-PCR for whitefly identification (table 2). The triplex real-time RT-PCR assays allowed the simultaneous and specific identification of all whitefly species. The results obtained were in accordance with morphological discrimination of species and molecular identification of species through PCR. The TaqMan probes selected were: BEM COI MEAM1-probe, BEM COI MED-probe, TRIAL COI-probe. In Sicilian greenhouses, all whiteflies col-

Table 3. Results of analysis of one step triplex real time RT-PCR using the three new specific probes and primers selected for the whitefly identification and the specific probes and primers for the detection of the two viruses in insects and in tomato leaves collected in greenhouse.

Greenhouse/ locality	Whitefly identification			Virus infections			
				in whiteflies		in tomato leaves*	
	T. vaporariorum	*B. tabaci* MEAM1	*B. tabaci* MED	ToCV	TICV	ToCV	TICV
1/ Vittoria (Sicily)	1/6	0/6	5/6	6/6	0/6	2/3	0/3
2/ Vittoria (Sicily)	0/32	0/32	32/32	25/32	0/32	2/9	0/9
3/ Acate (Sicily)	0/26	0/26	26/26	19/26	0/26	7/11	0/11
4/ Acate (Sicily)	0/31	0/31	31/31	8/31	0/31	4/11	0/11
5/ Arborea (Sardinia)	18/18	0/18	0/18	0/18	2/18	0/21	11/21
Total	19/113	0/113	94/113	58/113	2/113	15/55	11/55

* Leaves collected and analyzed for confirmation of the crinivirus infection in greenhouse.

lected belonged to *B. tabaci* MED, with an exception for an individual belonging to *T. vaporariorum*. No MEAM1 specimens were found. Fifty-eight insects showed positive to ToCV. All Sardinian samples belonged to *T. vaporariorum* and two specimens showed positive to TICV (table 3 and figure 1).

The assay was also applied to identify whitefly population at the early stages (nymphal and pupal stage) occurring on the leaves. As a consequence, twenty-six virus infected tomato leave samples were analyzed by Triplex TaqMan RT-PCR using the specific probes for whitefly identification (figure 1). Eleven samples showed infestation of *T. vaporariorum* (all collected in Sardinian greenhouse), ten samples indicated the presence of *B. tabaci* MED infestation and five were negative (all samples collected in Sicilian greenhouse).

Discussion

Whiteflies and the associated viral diseases become of increasing concern worldwide. Among whitefly-transmitted virus, TICV and ToCV raise particular interest for a different vector specificity as TICV is only transmitted by *T. vaporariorum* while ToCV is transmitted by either *Trialeurodes* spp. (*T. vaporariorum* and *T. abutiloneus*) or different species of the *B. tabaci* complex. This aspect greatly stimulated researches for a better knowledge on virus-vector relationships and epidemiological studies on distribution of TICV and ToCV according to whitefly-species populations in tomato crops in many parts of the world. In the past, many protocols based on different techniques were used to provide complete data on whitefly and virus identification, resulting time consuming and requiring different specialists for pests and pathogens. Molecular diagnostics based on amplification chain reaction (PCR), associated to restriction length polymorphism (RFLP) or random amplified polymorphic DNA analysis (RAPD) or DNA sequencing, introduced the advantage of rapidly distinguishing whitefly species, without morphological identification that require a well maintained specimens (Guirao *et al.*, 1997; Frohlich *et al.*, 1999; De Barro *et al.*, 2000; Bosco *et al.*, 2006), as well as genetic polymorphisms within species when morphologically indistinguishable. More recently, fluorogenic amplification assays (real-time PCR and real-time RT-PCR) were developed for identification of both virus in plant tissues and vectors (Cavalieri *et al.*, 2011; Papayiannis *et al.*, 2011; Tiberini *et al.*, 2011) and for MEAM1 and MED in *B. tabaci* (Jones *et al.*, 2008; Papayiannis *et al.*, 2009).

Moreover, in the above papers, total DNA and total RNA were extracted for whiteflies and viruses, respectively, causing an increasing of time and costs for the diagnostic process. In our investigation, total RNA extracts from insect were successfully used to identify either whitefly species or TICV and ToCV. Under this condition, the triplex real-time RT-PCR provided sensitive and accurate results in adults in which a specific discrimination between *T. vaporariorum*, *B. tabac*i MEAM1 and *B. tabaci* MED or TICV and ToCV were detected in the extract obtained from a single individual tested in the same amplification process. The Ct value (14-18) of 18S rRNA in the whitefly samples allowed to confirm the quality of the RNA extraction and the performance of the assay. Equally, accurate results were obtained in identifying whitefly species which nymphs and pupae were colonizing the infected tomato leaves used as RNA target in real-time RT-PCR assays (table 4). This may represent an advantageous diagnostic tool, that makes it unnecessary to collect whitefly adults or extract new RNA from these insects, since with a single infected and infested leaf sample it is possible to identify both the virus and their vectors.

Table 4. Threshold cycles range obtained in analysis of one step triplex real time RT-PCR using the three new specific probes and primers selected for the whitefly identification and the specific probes and primers for the detection of the two viruses within insects and tomato leaves collected in greenhouse.

Specific probes	Threshold cycles range	
	in insect samples	in plant samples
Whiteflies identification		
BEM COI MEAM1	21-22	-
BEM COI MED	18-29	20-38
TRIAL COI	18-35	19-37
Virus identification		
TICV	<38	<38
ToCV	<38	<38
Whitefly 18S	>14	-

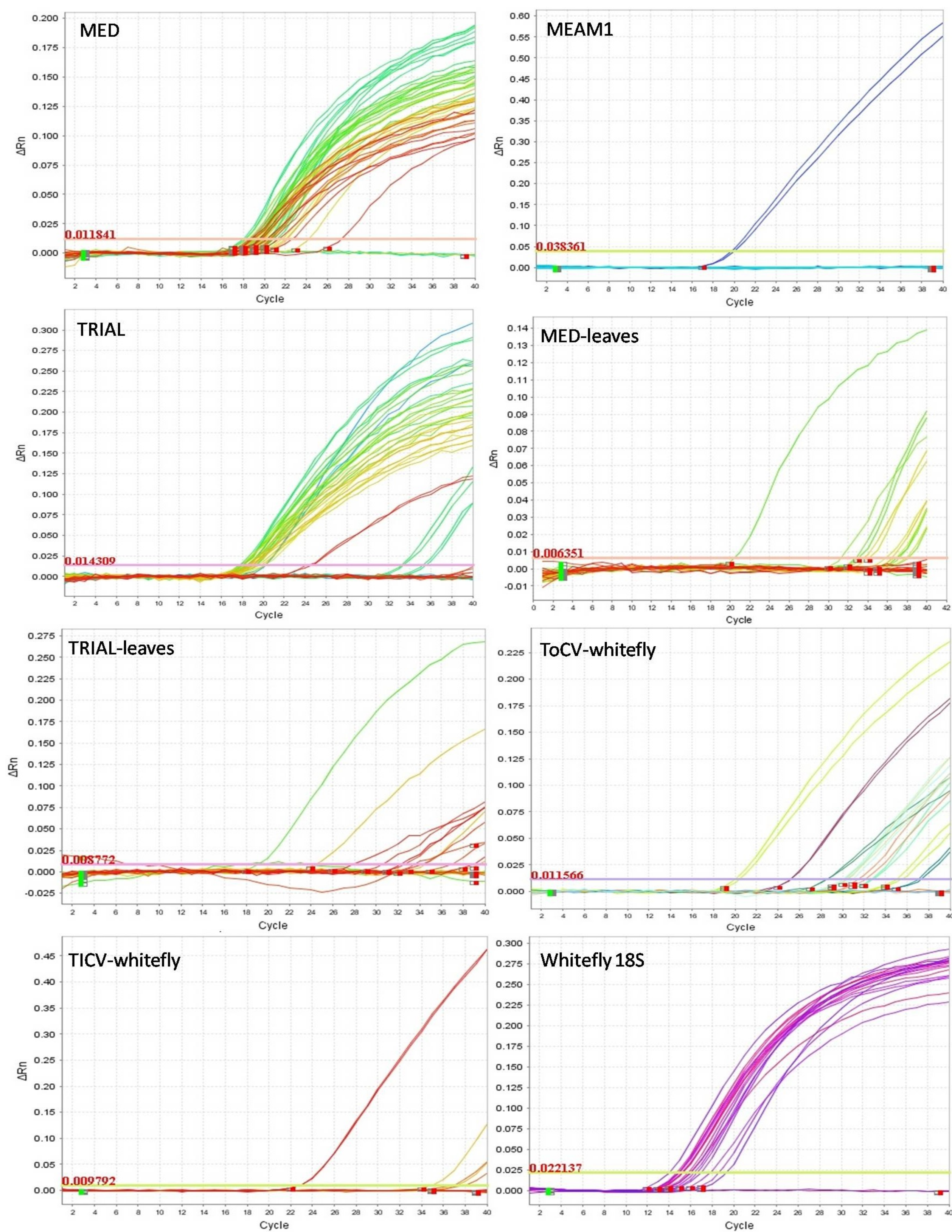

Figure 1. Triplex real time RT-PCR assays: amplification plots of: *B. tabaci* MED (MED); *B. tabaci* MEAM1 (MEAM1); *T. vaporariorum* (TRIAL); *B. tabaci* MED nymphs/pupae in infested leaves (MED-leaves); *T. vaporariorum* nymphs/pupae in infested leaves (TRIAL-leaves); ToCV in infected whiteflies (ToCV-whitefly); TICV in infected whiteflies (TICV-whitefly); whitefly 18S internal control (Whitefly 18S). All samples (a color for each sample) were amplified by specific probes and primers.

The absence of *B. tabaci* MEAM1 in our sampling could be due to the low number of investigated greenhouses and to the scarce number of specimens found on crops due to insecticide treatments against the moth *Tuta absoluta* (Meyrick), a new serious threat of Italian tomato crops. Similar reasons apply to the absence of *B. tabaci* within samples collected in Sardinia and the single specimen of *T. vaporariorum* collected in Sicily. The occurrence and distribution of different whitefly species influenced the presence of the two criniviruses in the investigated greenhouses. Indeed, in Sardinian greenhouses, where only *T. vaporariorum* was found, plants were infected only by TICV; on the contrary, in Sicilian greenhouses, where *B. tabaci* MED was exclusively present, tomato plants were infected only by ToCV.

In conclusion, the method here developed provides researchers and technicians with a reliable and sensitive (single specimens) tool by an accurate and unequivocal identification either of whitefly (adults and nymphs) species or the two criniviruses by a single analysis saving time and cost (the same extraction process for both whitefly and virus analysis; easily processed 96 sample per day, including setup and data analysis). Scientific studies on vectors and viruses interaction, epidemiology and spatial distribution could benefit from this protocol to increase knowledge in favor of control for whiteflies and the associated viral diseases.

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