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Full Length Article

Real-time qPCR Assay for the TYLCV Titer in Relation to Symptoms-Based Disease Severity Scales

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Abstract

During the last few decades, Tomato yellow leaf curl disease (TYLCD) has resulted in heavy yield losses and increased incidences in tropical and subtropical regions. In Oman, six different species of begomoviruses were found to be associated with TYLCD. However, *Tomato yellow leaf curl virus*- Oman (TYLCV-OM) was found to be the most widely distributed species in the whole country. A real-time quantitative PCR (qPCR) assay with an internal control [tomato elongation factor 1(EF1)] was developed for TYLCV-OM, based on SYBRGreen chemistry for the rapid detection and quantification of the virus. This assay was carried out on field samples showing symptoms according to The World Vegetable Center (AVRDC) disease severity scales. The test was used to establish a relationship between virus load and phenotypic symptoms. Viral copies of 2.88×10^9 were detected in field infected tomato plants showing symptom severity according to AVRDC scale '3' (*i.e.* severe leaf curling and yellowing of leaves). The copy number of virus decreased with the relative decrease in symptom severity scale. Tomato plants exhibiting scale '1' and '2' have 7.7×10^4 and 7.47×10^6 viral copies, respectively. Very low copy number of the virus (564) was detected in tomato plants showing symptoms at a severity scale of '0', which were apparently symptomless. However, tomato plants developed by tissue culture in sterilized conditions, which were used as a negative control, did not show the presence of the virus. The developed qPCR assay could detect as low as 18 fg (femto gram) of virus from total nucleic acid equivalent to approximately 30 genomic units. This quantitative assay can be used to determine virus titer, in breeding programs for development of virus resistant plants and for epidemiological surveys to monitor viral populations and their intensities. © 2017 Friends Science Publishers

Keywords: TYLCV; *Lycopersicon esculentum*

Introduction

Tomato fields are seriously affected by Tomato yellow leaf curl disease (TYLCD), which is one of the most destructive viral diseases worldwide. TYLCD has also become the major limiting factor for tomato production in different regions of the Middle East and Southeast Asia, Africa, Europe (Czosnek and Laterrot, 1997; Moriones and Navas-Castillo, 2000) and other countries (Accotto *et al.*, 2000, 2003). Several genetically-related virus species, which belong to the genus *Begomovirus* of the family *Geminiviridae*, are associated with TYLCD. TYLCD cause extensive crop losses that may reach up to 100%. Reduction in tomato production has been associated with TYLCD since it was first described in the late 1930s. Cohen and Harpaz (1964) reported TYLCD in the Jordan valley which has now become a serious problem infecting tomatoes worldwide. TYLCV alone or by mixed infections of different *Begomovirus* species is responsible for total yield loss in different countries. TYLCV is the generic name given to the complex of viral species that cause the disease. According to the International Committee on Taxonomy of Viruses (ICTV), a complex of more than 12 different viral species and their strains are found to be linked with TYLCD (Fauquet *et al.*, 2008). TYLCV is transmitted in a circulative, persistent manner by its vector whitefly (*Bimisia tabaci*) (Moriones and Navas-Castillo, 2000).

The genome of TYLCV is monopartite with a single genomic component, containing single-stranded DNA (ssDNA). The size of this ssDNA molecule is about 2.8 kb. Symptoms appear approximately 15 days after whitefly inoculation. Infected plants have small leaves that curl upward and turn yellow around the margins. Infected plants have short internodes, and stunted appearance. They show premature flower fall and loss of fruits. Fruits are small, but not misshaped. Infection of the plant at earlier stages results in a greater yield loss. Most of the wild tomato species, such as *Lycopersicon hirsutum, L. chilense, L. pimpinellifolium,*

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and *L. peruvianum* are symptomless carriers (Zakay *et al.*, 1991). In Oman TYLCD is associated with six begomoviruses but the most prevalent virus is TYLCV-OM which is found associated with almost all cases of the disease (Khan *et al.*, 2014).

The range of symptom severity on tomato plants infected with TYLCD under field conditions varies from mild to severe. The Asian Vegetable Research and Development Center (AVRDC), currently known as The World Vegetable Center (WVC), Taiwan has established a disease severity scale 0–3 (four scales) for recording symptom severity, where 0 = no visible symptoms; 1 = light leaf yellowing of the leaflet margins; 2 = moderate plant stunting with leaf yellowing and curling; and 3 = Severe plant stunting with leaf curling and yellowing and cessation of plant growth (Lapidot *et al.*, 1997; Lapidot and Friedmann, 2002). The scale is important in scoring susceptibility/resistance of tomato breeding lines under diverse climatic and growing conditions.

Highly sensitive tests are required for the detection and quantification of the virus in epidemiological studies to understand host range and virus-vector relationship. Moreover, these tests can also help in the selection of lines in breeding programs as well as in understanding the mechanism of resistance in resistant lines developed through conventional and genetic engineering approaches. Several methods have been established to detect and identify different *Begomovirus* species infecting tomato crops, like immunoblotting (Pico *et al.*, 1999), conventional polymerase chain reaction (PCR) methods, including species-specific primers and restriction fragment length polymorphism (Accotto *et al.*, 2000; Martínez-Culebras *et al.*, 2001; Davino *et al.*, 2008), loop-mediated isothermal amplification (Fukuta *et al.*, 2003) and real-time quantitative (q)PCR (Mason *et al.*, 2008). PCR is one of the methods used extensively for the detection of begomoviruses. The major drawback of conventional PCR is that it cannot determine the exact quantity of the virus. Moreover, some plants are phenotypically normal and carry very low virus titers which are below the detection limit of conventional PCR. So there is a need to quantify viruses in symptomatic as well as non-symptomatic plants.

The use of qPCR for the identification and quantification of DNA/RNA viruses has now become more attractive due to its greater accuracy and speed compared with conventional/end-point PCR or serological methods (Mason *et al.*, 2008; Papayiannis *et al.*, 2010). The use of an internal control in qPCR exhibits stable expression at various experimental conditions and allows normalization between samples. This kind of normalization is very effective in direct comparisons between independent samples and to avoid false negative results by removing any sampling, extraction or amplification bias that could hamper the analyses. In qPCR, several chemistries are available which include DNA binding dyes such as SYBR Green I, hybridization probes, hydrolysis probes and molecular beacon (Mullis and Faloona, 1987; Mullis, 1990; Tan *et al.*, 1994; Huang *et al.*, 1995a). Binding of the SYBER Green I to the minor grooves of double stranded (ds)DNA results in emission of fluorescence 1000 folds greater than its free form in solution (Huang *et al.*, 1995b). Thus, there is a direct correlation of fluorescence and synthesis of dsDNA in the reaction tube. This fluorescence can be determined which serves as the measure of amplified product. This method is relatively cheap and easy to use. Melt curve analyses can determine any nonspecific product during the reaction. In experiment, the fluorescence of the sample can be measured containing known DNA amounts in parallel to actual experimental reactions. The logarithm (log) of the amount of the starting quantities (SQ)versus threshold cycle (Ct) is used to plot standard curves to evaluate the reaction efficiency and to calculate the amount of DNA present in the experimental samples.

In this study, the enhanced sensitivity and specificity of qPCR was exploited for rapid detection and quantitation of TYLCV-OM using SYBR Green chemistry. This assay was then used on field infected tomato samples falling in different disease severity scales of AVRDC and the virus titer was correlated with symptom severity.

Materials and Methods

Sample Collection

Field samples infected with TYLCV-OM were collected in January 2014 from Al'Seeb wilayat of Muscat, Oman. The samples were categorized according to AVDRC disease severity scale 0–3 (Zhengxing, 1999). Ten samples of each scale were collected along with healthy tomato plants.

DNA Extraction for Template Preparation

Total DNA was extracted from 0.1 g fresh *Begomovirus*-infected plant leaves by CTAB method (Doyle and Doyle, 1987).

Primers Design

For conventional PCR, TYLCV-OM specific primer pair FC (GATGGGTTCCCCTGTGCGTGAATCCAT) and RC (GGACCAGCCTCCTCTTATAGAGAATAT) was used for the confirmation of TYLCV-OM infection in all selected samples with the amplified product of 300 bp designed on CP region. The primer pair QF-OM (GAAGCCCTGATGTTCCCCGTGG) and QR-OM (GATTTAACACAGAACCTCTTACC) was designed based on the CP gene of TYLCV-OM and was used in qPCR reactions. To validate and standardize qPCR, an internal control EF1 (EF1-F: TACTGGTGGTTTTGAAGCTG and EF1-R: AACTTCCTTCACGATTTCATCATA) was designed and used. Clustal X program (Thompson *et al.*, 1997) was used for multiple alignments.

Standard Preparation

For the absolute quantification of CP gene, a plasmid containing the full-length TYLCV-OM clone (Acc. No. DQ644565) was quantified with NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, USA) and serial dilutions were made to obtain a standard curve. The amount of viral DNA was used to calculate the copy number of the virus. The suitable amount of plasmid DNA was used to produce 1 X 10^9 copies of the virus and considered as stock. The stock sample was further 10-fold diluted to prepare five serial dilutions. A 10 ng μL^{-1} healthy tomato plant genomic DNA developed by tissue culture was also spiked in each serial dilution to get the same background as in field infected plants. All prepared standards were then aliquoted and frozen before being used as standards in each qPCR run. These serial dilutions were used to produce a standard curve. The threshold cycle (Ct) value of three replicates of each standard-dilution and the log of the total DNA in each sample were used to obtain the standard curves by linear regression analysis.

Real-time PCR Conditions

A qPCR assay was done in Applied Biosystems® 7500 Real-Time PCR Systems (Life technologies, USA). The qPCR reaction mix consisted of (10 µL) of 1X Power SYBR Green master mix, 0.10 µL of each primer and 2.5 µL of DNA sample (10 ng μL^{-1}). PCR reactions were carried out in clear optical plates in the ABI 7500 real time PCR detection system (USA).

Data Analysis

Data analysis was done using ABI 7500 software v 2.0.6 for each template sample individually. The analysis includes the calculation of all parameters of the standard curves and their corresponding dissociation curves and also calculates the Ct values for each sample individually.

Results

Infected Sample and Symptom Severity

During the field surveys, tomato plants exhibited a range of symptom severity (Fig. 1). The plants were categorized according to AVDRC disease severity scale (0–3). All plants showing symptom severity scale 1–3 upon PCR showed a band of 300bp indicative of the presence of *Begomovirus* infection. No amplification was observed in case of plants showing symptom severity scale 0 or healthy negative controls.

Primer Designing and Validation

The ClustalX program was used to make multiple sequence alignment of all TYLCV sequences available in the GenBank database reported from different geographical regions (Fig. 2). A list of viruses used in multiple alignment is given in Table 1. A highly conserved region in CP gene (co-ordinate 603-762) was selected for designing primers (Fig. 2). The amplicon size was 159 bp.

The other related and unrelated *Begomovirus* species were also checked by the primer QF-OM/QR-OM for their specificity and no amplification was obtained in any case either in conventional or qPCR assay (data not shown). More than 50 different TYLCV isolates from Oman were analyzed by multiple sequence alignment and indicated that all tested TYLCV sequences had a maximum of five nucleotide mismatches within the 159 bp of amplified region (Fig. 2).

The primer pair QF-OM/QR-OM was initially used to optimize qPCR profile and working concentrations on TYLCV-OM reference samples. For the optimization of the qPCR assays, various primer concentrations (2.5–10 pM) and annealing temperatures (50–65°C) were tested in a 15 µL reaction containing 2.5 µL (10 ng/uL) DNA template. 10 pM, 5 pM and 2.5 pM dilutions for both primer pairs were prepared for qPCR optimization. In case of QF-OM/QR-OM the primer concentration that yielded the highest reporter fluorescence with no dimer formations (100% efficiency) was 2.5 pM, while in case of the internal control primer pair EF1-F/EF1-R, the 10 pM concentration was found to be best. Melt curve analyses were studied in each run to see nonspecific amplifications. A single peak was observed in all cases indicative of the specific amplification.

Real-time qPCR Standard Curve

After optimization of all conditions, qPCR was programmed for 1 cycle at 94°C for 5 min, followed by 40 cycles each consisting of 30s at 94°C, 30s at 55°C and 30s at 72°C followed by a melt curve analysis starting from 55°C. Triplicate reactions of each sample were run. Each qPCR reaction was repeated thrice to ensure the reproducibility of results. The actual function of developed qPCR assay was the construction of quantification standard containing the five viral serial dilutions and the internal reporter gene. The plasmid containing full-length TYLCV-OM clone was quantified by nanodrop. The quantified plasmid was then serially diluted in 10-fold steps from 10^9 to 10^3 copies per µL. The Ct was automatically calculated by the ABI 7500 software. The efficiency of the reaction from the total nucleic acid (TNA) extracts was slightly higher (98.58%) than plasmid forms.

The Ct values of five10-fold serial dilution of TYLCV-OM plasmid was used to construct a standard curve, demonstrating the sensitivity and linearity of the technique. The Ct values were relative to the log starting quantity of template DNA and the qPCR efficiency. Plasmid and TNA from tomato plants infected with TYLCD was estimated by the formula E = [10−1/slope]–1, were 97.8% (slope: −3.367) and 98.58% (slope: −3.353), respectively.

Table 1: Accession numbers and origin of Begomoviruses used in the multiple sequence alignment

Accession No.	Acronym	Virus species	Origin
JN604488	TYLCV-[OM-DT2-12]	TYLCV	Mediterranean/Middle East, Iran
DQ644565	TYLCV-[OM-Alb-09]	*ToLCABV	Oman
FJ956700	TYLCV-[OM-Alb22-11]	*ToLCABV	Oman
FJ956701	TYLCV-[OM-Alb12-11]	*ToLCABV	Oman
FJ956703	TYLCV-[OM-Alb23-11]	*ToLCABV	Oman
FJ956704	TYLCV-[OM-Alb26-11]	*ToLCABV	Oman
FJ956705	TYLCV-[OM-Alb34-11]	*ToLCABV	Oman
JN604484	TYLCV-[OM-KW1-12]	TYLCV	Mediterranean/Middle East, Iran
JN604485	TYLCV-[OM-KW2-12]	TYLCV	Mediterranean/Middle East, Iran
JN604486	TYLCV-[OM-KW3-12]	TYLCV	Mediterranean/Middle East, Iran
JN604487	TYLCV-[OM-DT1-12]	TYLCV	Mediterranean/Middle East, Iran

*Novel recombinant specie of ToLCOMV and ChLCV-OM isolated from tomatoes in Oman

Fig. 1: Field infected tomato plants showing typical TYLCV-OM symptoms (leaf curling, stunting and yellowing). The plants were categorized according to AVDRC disease severity scale: 0: Normal healthy plant 1: light leaf yellowing, 2: moderate plant stunting with leaf yellowing and curling, 3: Severe plant stunting with leaf curling and yellowing. Panels A-D represent scale 0-3, respectively

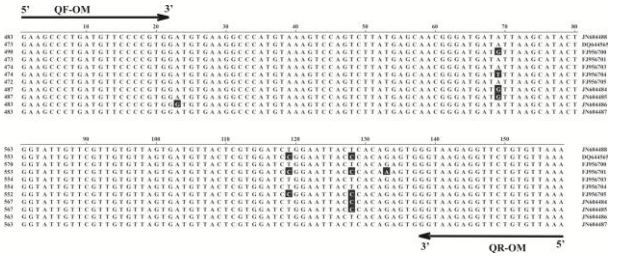

Fig. 2: Multiple sequence alignment of 11tomato yellow leaf curl disease isolates using CLUSTALW. The highly conserved region of coat protein (CP) gene of TYLCV-OM was selected. The accession no and coordinates of the isolates used to design primer are indicated. The arrows represent the length of primers

Sensitivity of Conventional and qPCR

The TYLCV-OM infected tomato DNA sample and TYLCV-OM plasmid DNA was used to compare the sensitivity of qPCR with conventional PCR. A 10-fold serial dilution of TNA (150 ng, 15 ng, 1.5 ng, 150 pg, 15 pg, 150 fg, 15 fg, 1.5 fg) from TYLCV-OM infected tomato plant was used as a template in qPCR and conventional PCR reactions. The minimum possible dilution accurately detected by conventional PCR was 150 pg, whereas qPCR was able to detect the last dilution point of 1.5 fg with Ct of 38.34. The qPCR reaction with 15 fg of template DNA was detected at a Ct of 34.95. In case of plasmid DNA, conventional PCR was limited to detect 3000 genomic units; whereas qPCR detected as low as 30 genomic units. It was inferred from the observed results that the sensitivity of qPCR assay in detecting serial dilutions was 1000 times more ($1:10^{-8}$) than the conventional PCR ($1:10^{-5}$; Fig. 3).

Symptom Severity Scale and Virus Quantification

Before proceeding to qPCR, all field collected samples were first checked for the presence of TYLCV-OM by conventional PCR using the primer set FC and FR. All samples showing symptom severity scales of 1-3 showed the presence of TYLCV-OM, while no amplification was observed in case of samples showing a disease severity scale of 0 (Table 2). These results confirmed that TYLCV-OM is the prevalent virus and is present in all infected tomato plants in Oman. Five samples from each scale were selected for qPCR analysis. Each sample was run in five replicates.

The Ct values for scale 1were more than scale 2 and 3 (Fig. 4). Virus copies were detected in small amounts in tomato plants showing a symptom severity scale of 0. The amount of virus copies associated with samples from scale 0 was significantly lower (564) than other scales (Fig. 5). However, negative controls (DNA of healthy tomato developed by tissue culture) did not show any virus with the Ct value of 37.414 (Fig. 4 and Fig. 5). The Ct values were proportional to the log of starting quantity of template DNA and SYBR Green PCR efficiencies for the TYLCV-OM assays was 99.5% (slope: −3.884). The average value of virus detected for scale 0–3 ranged from 564–2.88×10^{9} with the Ct values ranged from 37–8, respectively.

Table 2: PCR-based detection of TYLCV-OM in tomato plants differing in the severity of TYLCV

AVRDC severity Scale	Sample size	PCR for TYLCV-OM
S0	10	-
S1	10	+
S2	10	+
S3	10	+

The (+) sign indicates detection of the virus while the (-) sign indicates that the virus was not detected using PCR

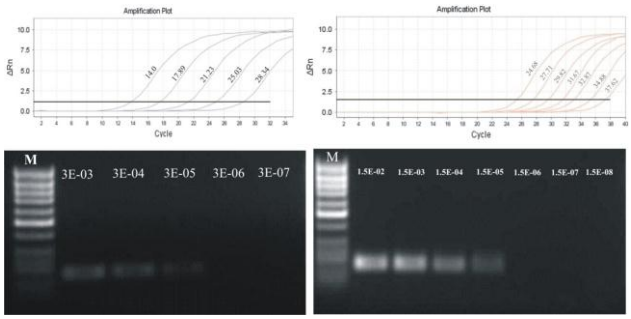

Fig. 3: Real-time quantitative PCR to quantify viral DNA in field infected tomato plants with TYLCV-OM. The assay was carried out in all four groups of plants belonging to scale 0-3. A blue bar represents the viral DNA concentration in plants. Vertical axis represents the no virus particles starting from 100, 000, 0-100, 000, 000, 0. Each bar is the mean of three replicates and the error bars indicate standard deviation

There was a positive correlation between disease severity scale and virus titer. Significant differences were found for viral accumulation between the different scales (Fig. 4). The field samples of scale 0 did not exhibit any visual symptoms (Fig. 1). Although conventional PCR fails to detect any virus in those sample but qPCR showed appreciable amount of viral DNA, yet 1000 fold less than scale 1 (Fig. 5). These results showed that apparently healthy field samples had enough virus inoculum for the white flies to take up. The difference in viral copies of plants from scale 1 and 2 was relatively less as compared to other scales. The highest amount of virus titer was found in scale 3 with values reaching up to 2.88 x 10^9 (Fig. 5).

Discussion

TYLCD has become the major limiting factor for tomato production in many warm and temperate regions worldwide by developing new recombinants. According to ICTV, TYLCD is a disease complex comprised of several different virus isolates/species which belong to the genus *Begomovirus*. Among various isolates responsible to TYLCD complex, currently TYLCV isolate has been reported as the most important *Begomovirus* species infecting *Solanaceous* and other vegetable crops in Europe and the Mediterranean Basin.

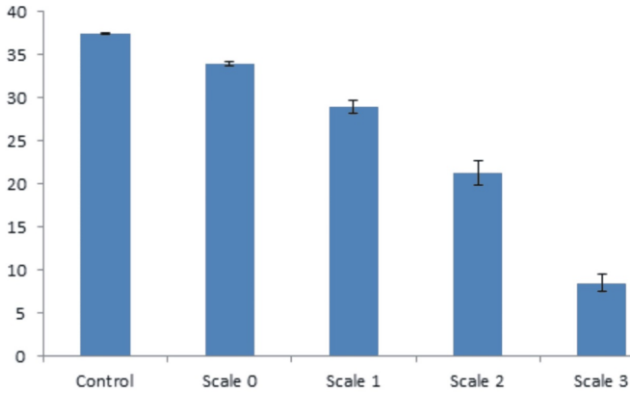

Fig. 4: CT mean value of 4 sets of plants calculated from qPCR. Each bar is the mean of five replicates and the error bars indicate standard deviation

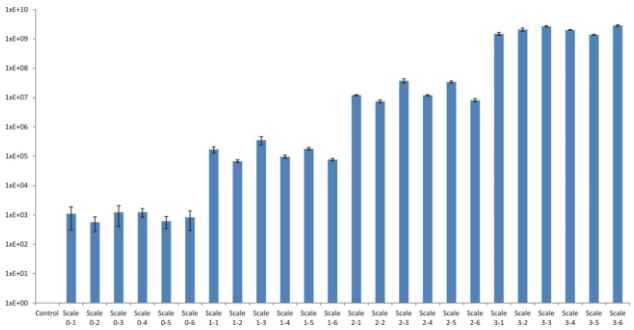

Fig. 5: Comparison of PCR and qPCR sensitivity based on (A) circular plasmid and (B) total nucleic acid (TNA) extracts of TYLCV-OM infected tomatoes. The same concentrations of plasmid genomic units were used in both assays and are labeled on gels. The Cq numbers denoted on amplification curves are means of duplicate runs. M: 1 kb molecular marker

It is also believed that TYLCV has emerged somewhere in the Middle East and extends throughout the Mediterranean basin, Asia, Africa and America (Navas-Castillo *et al.*, 1999; Accotto *et al.*, 2003; Papayiannis *et al.*, 2010; Davino *et al.*, 2012; Khan *et al.*, 2014).

The recent advancement of molecular diagnostic systems during the past few years has enabled rapid diagnosis of TYLCD in field. Similarly several conventional PCR and qPCR-based techniques have improved remarkably for the detection of various virus species (Accotto *et al.*, 2000; Gorsane *et al.*, 2005; Davino *et al.*, 2009). The development of fluorescent methods for PCR and also the instruments like real-time which can monitor the amplification process is considered as a significant improvement in molecular biology. qPCR have been extensively used in various branches of life sciences during the past few years, which includes infectious disease detection in humans (Enbom *et al.*, 2001), animals (Brinkhof *et al.*, 2008) and plants (Boonham *et al.*, 2004),

differentiation of invertebrate biotypes within a species (Papayiannis *et al.*, 2009) and mutation scanning (Wittwer *et al.*, 2003).

In the present study a qPCR assay based on SYBR Green chemistry was developed for the detection and quantification of TYLCV-OM and successfully applied on TYLCD affected tomato plants from a field showing different severity scales. The data presented here showed the sensitive and specific detection/amplification of TYLCV-OM and there was a positive correlation between virus titer and symptom severity. During the development of this assay, it was ensured that no cross-reaction occurred between different TYLCV species isolated from Oman. The developed assay was also compared with conventional PCR for its sensitivity. Results showed that qPCR assay developed in this study can detect virus down to a dilution of $1:10^{-8}$; 1000 times more sensitive than conventional PCR. Our results have shown that plants from symptom severity scale 0, which were asymptomatic, carry small amount of the virus. Conventional PCR failed to detect virus however, qPCR was sensitive enough to detect such low virus copies in plants showing a symptom severity scale of 0. These results are highly significant and indicate that TYLCD is present in the filed in apparently healthy plants. These plants though remain symptomless but can serve as a reservoir of viruses where their vector can carry and transmit these viruses to other hosts. The greater specificity and sensitivity of developed qPCR assay can thus be used in such samples/hosts where conventional PCR fail to detect virus as shown in this study.

In the present study, we have developed a specific qPCR method for detection/quantification of TYLCV-OM and the relative amount of virus levels in field infected plants has been evaluated. The developed assay can also be used for other related hosts of TYLCV-OM from *Solanaceae* family like pepper, chili, tobacco and alternative hosts like weeds. The practical use of hazardous compounds like ethidium bromide for the pre- and post-amplification detection by gel electrophoresis could be minimized by using the assay developed in this study. Moreover, this method can be used to detect and quantify virus in those hosts where virus titer is lower than the detection limit of conventional methods. Therefore, this assay can be used in breeding programs for the identification of sources of resistant and for epidemiological surveys to monitor viral spread and population. It is speculated that all worldwide TYLCV isolates which share similar or identical nucleotide mismatches could be accurately detected and quantified by the developed TYLCV qPCR assay.

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