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ORIGINAL ARTICLE

Multiplex PCR methods for simultaneous detection of tomato brown rugose fruit virus, tomato spotted wilt virus and pepino mosaic virus

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Abstract

Tomato (*Solanum lycopersicum*) is a crucial vegetable globally, pivotal in securing the world's food supply. However, viral diseases pose a significant threat to tomato cultivation, resulting in considerable yield losses. Employing sensitive, rapid, and cost-effective detection methods is imperative for mitigating such losses in agricultural production. In response, we developed a Taqman® probe-based real-time multiplex PCR method capable of concurrently detecting three major plant viruses: Tomato brown rugose fruit virus (ToBRFV), tomato spotted wilt orthotospovirus (TSWV), and pepino mosaic virus (PepMV), all of which are prominent viral pathogens affecting tomato production. Utilizing envelope protein encoding sequences of these pathogens, we designed primers and probes, assigning the FAM reporter for ToBRFV, the HEX reporter for TSWV, and the Cy5 reporter for PepMV detection. The multiplex RT-qPCR experiment yielded successful results, with Ct values of 29.34, 25.93, and 27.47 for ToBRFV (at 10^{-6} dilution), TSWV (at 10^{-2} dilution), and PepMV (at 10^{-3} dilution), respectively. Developed primers facilitate the early detection of destructive pathogens ToBRFV, TSWV, and PepMV using both RT-PCR and RT-qPCR, offering a user-friendly and cost-effective approach.

KEYWORDS
multiplex RT-qPCR, PepMV, ToBRFV, tomato, TSWV, virus

1 | INTRODUCTION

Tomato (*Solanum lycopersicum*) is essential crops globally, serving as a staple food source and contributing significantly to agricultural economies. However, tomato cultivation faces formidable challenges, chiefly from viral diseases that inflict substantial yield losses (Jeger, 2020). Notably, tomato spotted wilt orthotospovirus (TSWV), pepino mosaic virus (PepMV), and tomato brown rugose fruit virus (ToBRFV) are among the most prevalent viral pathogens affecting tomato production (Karimova et al., 2021).

Tomato brown rugose fruit virus, originally identified in Israel and subsequently disseminated worldwide, emerged in Turkey in 2019, presenting a significant threat to tomato production. Its presence has profoundly affected tomato yields, depreciating market values and necessitating the removal of infected fruits from greenhouses (Fidan et al., 2019). Notably, TSWV, PepMV, and ToBRFV are designated as quarantine pathogens in numerous countries, including Turkey, a prominent exporter of tomatoes (Fidan et al., 2021; Ozdemir, 2010; Şevik, 2011).

Within the scope of the SPS notification made by the Russian Federation to the World Trade Organization, analysis of ToBRFV,

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TABLE 3 Primer-probe sequences designed for RealTime PCR.

Primer	Sequence (5′–3′)	Reporter dye	Quencher	Annealing	PCR product
ToBRFV F	ACTAGGTAATCAGTTCCAAACA	FAM	BHQ1	60°C	149 bp
ToBRFV R	GGACAGGTTTCCACACTT				
ToBRFV P	AGCTAGAACAACCGTTCAACGGCA				
TSWV F	CTAAGATTGCTTCTCACCCTCT	HEX	BHQ1	60°C	98 bp
TSWV R	AAGCTACCTCCCAGCATTAT				
TSWV P	TGATGCAAAGTCTGTGAGGCTTGC				
PepMV F	CTAAGATTGCTTCTCACCCTCT	CY5	BHQ2	60°C	112 bp
PepMV R	AAGCTACCTCCCAGCATTAT				
PepMV P	TGATGCAAAGTCTGTGAGGCTTGC				

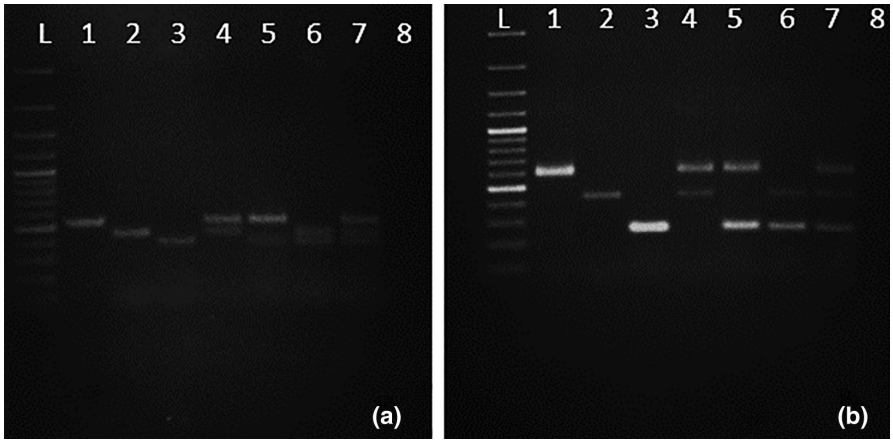


FIGURE 3 RT-PCR results. (a) (1) PepMV2, (2) ToBRFV2, (3) TSWV, (4) ToBRFV2 + PepMV2, (5) TSWV + PepMV2, (6) TSWV + ToBRFV2, (7) PepMV2 + TSWV + ToBRFV2, (8) single, duplex and multiplex analysis results with negative control primers; (b) (1) PepMV1, (2) TSWV and (3) ToBRFV1, (4) PepMV1 + TSWV, (5) PepMV1 + ToBRFV1, (6) TSWV + ToBRFV1, (7) PepMv1, ToBRFV, (8) single, duplex and multiplex analysis results with negative control primers.

TABLE 4 Ct values of the dilution series for each virus and channel.

Example	FAM (ToBRFV)	HEX (TSWV)	Cy5 (PepMV)
Stock TNA	16.93	18.61	19.47
10^{-1}	18.05	22.28	20.40
10^{-2}	20.86	25.93	23.75
10^{-3}	22.95	32.99	27.47
10^{-4}	25.05	36.55	31.17
10^{-5}	27.12	39.86	35.77
10^{-6}	29.34	–	36.89
10^{-7}	33.32	–	38.23
10^{-8}	35.77	–	39.88
10^{-9}	39.81	–	40.81
Nalan F1	40.75	40.08	41.19

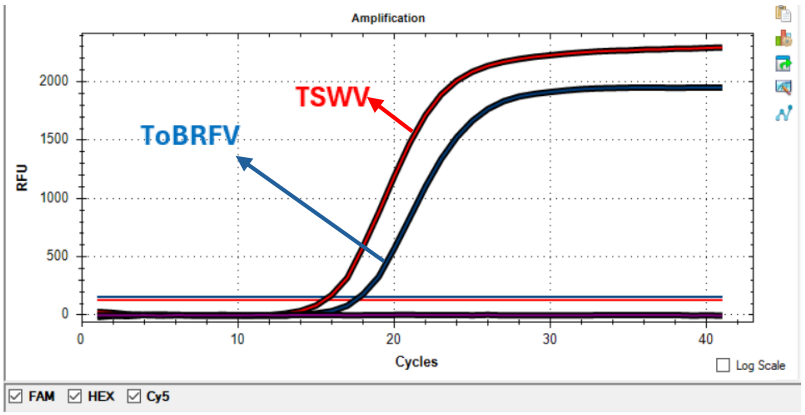
enveloped, and nonenveloped), which can aid in distinguishing between different virus genera (Richert-Pöggeler et al., 2019).

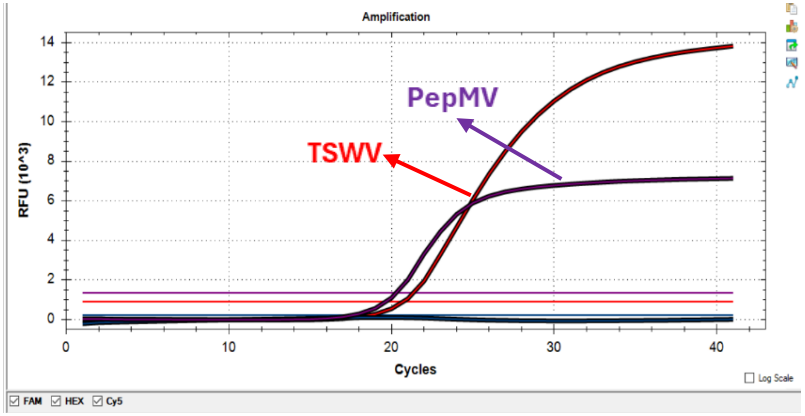
In contrast, molecular approaches offer greater reliability by targeting specific regions of virus DNA and RNA genomes using designed primers and probes (Cassedy et al., 2021). Moreover, multiplex assays are advantageous due to their user-friendly nature, cost-effectiveness, and reduced time requirements compared to traditional methods (Cao et al., 2023). They enable the simultaneous detection of multiple plant pathogens, including viruses, in a single reaction, making them highly efficient tools for disease diagnosis and management (Jangra et al., 2020). Therefore, in this study, we focused on the development of a Multiplex RT-qPCR and RT-PCR-based detection method for ToBRFV, TSWV, and PepMV, which infect tomatoes.

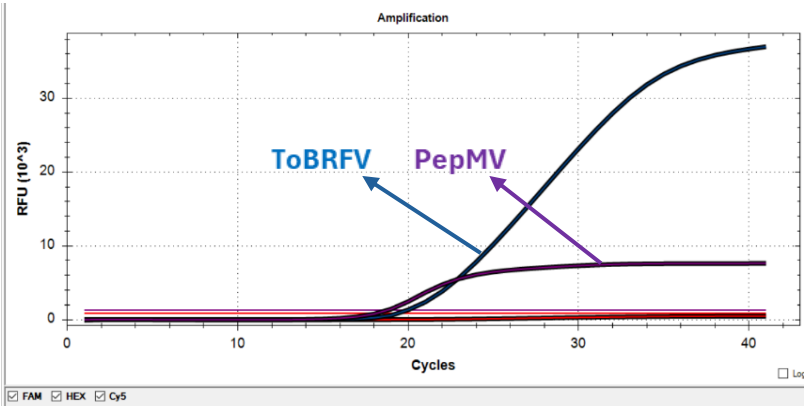
The developed primers demonstrated success in detecting ToBRFV, TSWV, and PepMV in multiplex RT-PCR, even in triple reactions simultaneously. The product sizes of the RT-PCR products were distinct and discernible from each other, whether in double combinations (ToBRFV-TSWV, ToBRFV-PepMV, or TSWV-PepMV) or triple combinations (ToBRFV-TSWV-PepMV), or even in single reactions. While electrophoresis and visualization of RT-PCR products can be time-consuming, the developed primers offer utility, especially in laboratories lacking qPCR equipment or resources.

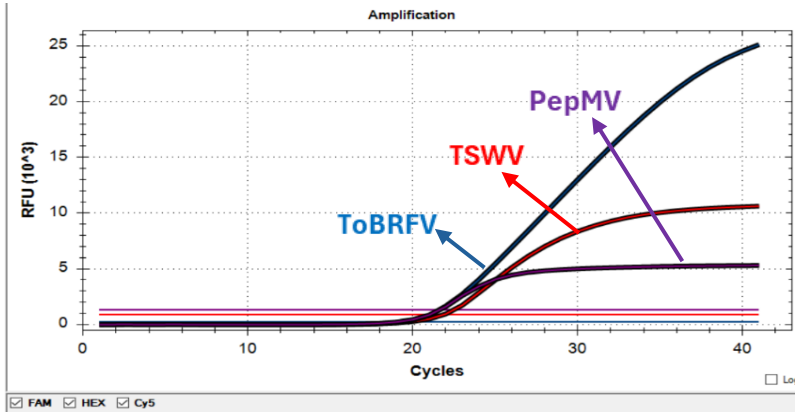
Similarly, multiplex-based methods have been developed for the detection of PepMV and *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Additionally, Chen et al. (2011) developed multiplex primers for TMV and ToMV, while Dai et al. (2012) devised a multiplex

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Amplification
TSWV
ToBRFV
RFU
2000
1500
1000
500
0
0
10
20
30
40
Cycles
Log Scale
FAM
HEX
Cy5


Amplification
PepMV
TSWV
RFU (10^3)
14
12
10
8
6
4
2
0
0
10
20
30
40
Cycles
Log Scale
FAM
HEX
Cy5


Amplification
ToBRFV
PepMV
RFU (10^3)
30
20
10
0
0
10
20
30
40
Cycles
FAM
HEX
Cy5


Amplification
PepMV
TSWV
ToBRFV
RFU (10^3)
25
20
15
10
5
0
0
10
20
30
40
Cycles
FAM
HEX
Cy5