Detection of Zucchini yellow mosaic virus infecting pumpkin using realtime RT-PCR

Nhi H. Lu[1], Toan Q. Truong[2], & Biet V. Huynh[1,2*]

[1]Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: August 31, 2024
Revised: November 02, 2024
Accepted: November 22, 2024

Keywords

Curcubita moschata
Realtime RT-PCR
Yellow mosaic
ZYMV

***Corresponding author**

Huynh Van Biet
Email: hvbiet@hcmuaf.edu.vn

ABSTRACT

Zucchini yellow mosaic virus (ZYMV) is a significant pathogen causing yellow mosaic disease in *Cucurbitaceae*. It can spread rapidly from infected plants to healthy ones or through contaminated seed sources, leading to a substantial reduction in the yield and quality of pumpkins after harvest. Currently, there is no effective treatment to eliminate this virus, making seed screening prior to planting and the removal of symptomatic plants the most effective control methods. In this study, a 214 bp target gene of the ZYMV was amplified using specific primers, then cloned into the pJET1.2 vector and transformed into *Escherichia coli* JM109. A realtime RT-PCR procedure was developed to detect and quantify ZYMV utilizing a primer pair designed for a 164 bp product. The standard curve was established with the equation y = -3.417x + 49.605 and correlation coefficient R^2 = 0.9969 for quantifying the ZYMV virus. The realtime RT-PCR was built with qualitative results corresponding to the PCR method. Additionally, the procedure quantified test samples with viral loads ranging from 7.1 x 10^6 to 8.5 x 10^9 copies/µL.

Cited as: Lu, N. H., Truong, T. Q., & Huynh, B. V. (2024). Detection of Zucchini yellow mosaic virus infecting pumpkin using realtime RT-PCR. *The Journal of Agriculture and Development* 23(Special issue 2), 183-192.

1. Introduction

Pumpkin (*Cucurbita moschata*) originates from Southern Mexico - Central America and is widely cultivated around the world, especially in subtropical areas such as Asia, America, and Africa (Lim, 2012). Often referred to as a "golden food", pumpkin is rich in vitamins and nutrients essential for human health. However, hot, dry weather or erratic rainfall can lead to infections by harmful diseases, including wilt (caused by *Pseudomonas solanacearum*), anthracnose (caused by *Colletotrichum lagenarium*), powdery mildew (caused by *Erysiphe cichoracearum*), leaf mosaic (caused by Squash mosaic virus), and yellow mosaic (caused by Zucchini yellow mosaic virus). Among them, yellow mosaic disease is particularly impactful, significantly reducing

both productivity and fruit quality a disease that significantly reduces productivity and fruit quality. The earlier the disease manifests, the lower the pumpkin yield at harvest. Research by Moradi et al. (2019) indicates that Zucchini yellow mosaic virus (ZYMV) is one of the most diverse strains of Potyvirus, capable of causing yield losses of up to 100%. This disease is primarily transmitted by aphids. Common symptoms include swollen leaves, yellow spots, reduced size, swollen buds, few fruits and deformed fruits, lumps, spots, cracks, reducing productivity and shelf life. Some symptoms may not be visibly apparent and can only be detected through molecular testing. However, in Vietnam, research on pumpkin diseases has been limited. Common diagnostic methods include ELISA, PCR, and realtime RT-PCR. While ELISA is simple and user-friendly, it lacks high sensitivity and can be time-consuming (Hu et al., 1993). The PCR is also widely used to diagnose viral diseases but this method is only capable of qualitative function and cannot detect virus-infected samples at low concentrations. Currently, the realtime RT-PCR method is considered optimal, as it can accurately quantify and detect pathogens at low concentrations with a quick turnaround time. Therefore, developing a realtime RT-PCR process for detecting the ZYMV is essential for early pathogen detection and timely prevention, helping to reduce the damage caused by this pathogen to farmers.

2. Materials and Methods

2.1. Materials

Pumpkin leaf samples were randomly collected from gardens in Tay Ninh province. After collection, leaf samples were stored at -20^{o} C until used for extraction. Each sample is placed in a separate bag and labeled with its corresponding name.

The RNA control sample for the ZYMV virus was provided by the Molecular Biology Laboratory at the Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam. *Escherichia coli JM109* virus strain was used for gene cloning.

2.2. RNA extraction and synthesizing cDNA from RNA

Total RNA was extracted from 50 mg of leaf sample using the EZ-10 Spin Column Plant RNA Miniprep Kit (Biobasic) following the manufacturer's protocol. The purity of RNA was assessed by measuring the optical density (OD) at 260 nm and 280 nm using a spectrophotometer (Biodrop, UK).

cDNA synthesis reactions were carried out according to the manufacturer's instructions for the SensiFAST™ cDNA Synthesis Kit (Bioline, UK). The reaction was conducted in a PCR machine (Applied Biosystems 2720 Thermal Cycler), the following thermal cycling conditions: 25°C for 10 min, 42°C for 15 min, 48°C for 15 min, and 85°C for 5 min.

2.3. Primers used in the study

Primers used for realtime RT-PCR to detect ZYMV virus were designed using Primer3 software to amplify a 164 bp gene segment (Table 1).

Table 1. Primers information

Primer name	Sequence 5' – 3'	Size (bp)	Source
ZYMVF	CATACATGCCGAGGTATGGTTT	214	Aguiar et al. (2019)
ZYMVR	GTGTGCCGTTCAGTGTCTT		
ZYMVF new	GGCTCGATACGCTTTCGACTT	164	This study
ZYMVR new	TGTGCCGTTCAGTGTCTTCG		
pJET1.2-F	CGACTCACTATAGGGAGAGCCGC	118	CloneJET ™ PCR Cloning Kit
pJET1.2-R	AAGAAC ATCGATTTTCCATGGCAG		

The sequence of primers was checked for the non-specific binding in NCBI Primer Blast tool. This primer pair had a high specificity to only pair with ZYMV (Figure 1).

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGCTCGATACGCTTTCGACTT	21	60.80	52.38	6.00	1.00
Reverse primer	TGTGCCGTTCAGTGTCTTCG	20	60.87	55.00	3.00	2.00

Products on target templates

>LC781588.1 Zucchini yellow mosaic virus OGC3 gene for coat protein, partial cds

```
product length = 164
Forward primer  1    GGCTCGATACGCTTTCGACTT  21
Template        419  ......................  439

Reverse primer  1    TGTGCCGTTCAGTGTCTTCG  20
Template        582  ......................  563
```

>LC781584.1 Zucchini yellow mosaic virus OGC5 gene for coat protein, partial cds

```
product length = 164
Forward primer  1    GGCTCGATACGCTTTCGACTT  21
Template        428  ......................  448

Reverse primer  1    TGTGCCGTTCAGTGTCTTCG  20
Template        591  ......................  572
```

>OP771419.1 Zucchini yellow mosaic virus isolate BY01 coat protein gene, partial cds

```
product length = 164
Forward primer  1   GGCTCGATACGCTTTCGACTT  21
Template        78  ......................  98

Reverse primer  1    TGTGCCGTTCAGTGTCTTCG  20
Template        241  ......................  222
```

Figure 1. The result of the primer was compared to the target sequences by using Primer Blast tool on NCBI Genbank.

2.4. PCR reaction process

The reaction was conducted in a total volume of 25 µL, comprising 12.5 µL of MyTaq Mix (2X) (Bioline, UK), 9.5 µL of nuclease-free water (Bio Basic, Canada), 0.5 µL (10 µM) of each forward and reverse primer, and 2 µL of the cDNA sample. The reaction was performed in a PCR machine (Applied Biosystems 2720 Thermal Cycler) and included three stages: Stage 1 with 94°C for 5 min, stage 2 consist of 35 cycles with 95°C for 30 sec, 52°C for 1 min 30 sec, 72°C for 2 min, stage 3 with 72°C for 8 min and hold at 4°C.

The amplified gene segment was sequenced at Nam Khoa Trading and Service Company Limited in Ho Chi Minh City. The sequencing results were then compared with the ZYMV virus sequences published on GenBank (NCBI).

2.5. Procedure for creating plasmid clones carrying target genes

The target gene segment of ZYMV virus was inserted into vector pJET1.2 according to the instructions of CloneJET ™ PCR Cloning Kit (Thermo Scientific). Following the insertion, the vector is transformed into *E. coli* JM109 bacteria using the chemical transformation method (Sambrook, 2001). After completing the cloning steps, the bacteria were cultured in an Luria-Bertani (LB) medium supplemented with 50 mg/L of Ampicillin.

After 24 h of bacterial culture, colonies growing on solid LB medium were selected for PCR reactions using the pJET1.2 primer pair (Table 1), following the instructions of the CloneJET PCR Cloning Kit (Thermo Scientific). The PCR products were then analyzed by electrophoresis.

The plasmid DNA sample obtained after amplification was sent for sequencing at Nam Khoa Trading and Service Company Limited, Ho Chi Minh City. Sequencing results were compared with the ZYMV virus sequence Prior to cloning, as well as with sequences published on GenBank.

2.6. Generation of a standard curve

Each reaction has a total volume of 10 µL, including 5 µL SensiFAST SYBR® Lo-ROX Kit (2X), 3.6 µL of nuclease-free water (Bio Basic, Canada), 0.2 µL (10 µM) of each forward and reverse primer, and 1 µL of the cDNA sample. Realtime RT-PCR reaction was performed using an Applied Biosystem 7500 Realtime PCR machine (Applied Biosystem, USA). The reaction comprised two stages: Stage 1: 95°C for 2 min, Stage 2: 40 cycles: 95°C for 15 sec, 60°C for 35 sec.

Plasmid DNA copy number was calculated using the formula (Staroscik, 2004):

$$\text{Number of copies} = \frac{6{,}022 \times 10^{23} \times C}{650 \times 10^{9} \times L}$$

Where: C is the DNA amount (ng), L is the length of the DNA (bp)

The plasmid DNA sample was diluted with nuclease-free water. Two parameters were used to evaluate the stability of the standard curve: the correlation coefficient R2 and amplification efficiency (PCR efficiency) E%. An R2 coefficient of 0.99 or higher indicates high linearity of the standard curve. The E% is accepted in the range of 90% to 110% and is calculated using the formula: E% = (E – 1) 100%; $E = 10^{-1/\text{slope}}$. The allowable slope of the standard curve ranges from -3.58 to -3.1, with an ideal slope of = -3.32. The standard curve can be expressed as Y = aX + b, where Y is the threshold cycle (ct) and X is the logarithm of the copy number, a is the slope, and b is the y-intercept.

2.7. Evaluation of the specificity of the Realtime RT-PCR

The cDNA of other *Potyvirus* including DsMV (Dasheen mosaic virus), PRSV (Papaya ringspot virus) and ChiVMV (Chilli veinal mottle virus) were tested with ZYMV primer to verify the specificity of the RT-PCR assay.

2.8. Applying the ZYMV virus detection process on samples collected from the field

Field samples were randomly collected to detect and quantify the level of infection. Samples were obtained from 2 gardens: garden 1 in Chau Thanh district, Tay Ninh city, and garden 2 in Ninh Son district, Tay Ninh city. The composition and thermal cycling conditions of the reaction were consistent those used to

generate the standard curve. The copy number of ZYMV - infected samples was determined using this standard curve.

3. Results and Discussion

3.1. PCR reaction results

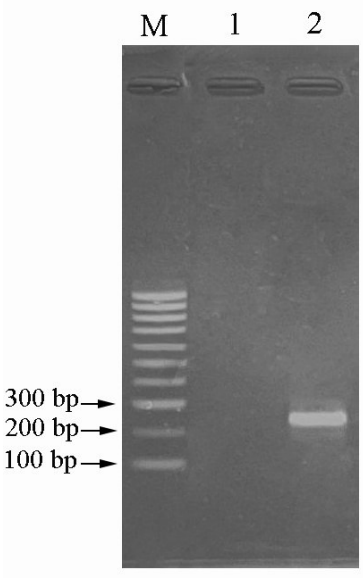

Figure 2. The electrophoresis results of the PCR product for the target gene segment using the primer pair Zucchini yellow mosaic virus F/R . M: DNA ladder, Well 1: negative control, Well 2: positive control.

The electrophoresis result (Figure 2) indicated that the sample in well 2 was positioned near the 200 bp band of the DNA ladder, corresponding to the amplification of the 214 bp gene segment (Aguiar et al., 2019). A comparison of the obtained sequence with ZYMV virus sequences published on GenBank revealed that the amplified gene segment is 99.07% similar to the ZYMV virus gene sequence with accession number DQ925448.1 (Ha et al., 2008). This confirms that the PCR reaction established in this study successfully amplified the correct gene segment of the ZYMV virus.

3.2. Cloning a bacterial strain carrying the ZYMV gene

Figure 3. White colonies grow on Luria-Bertani medium.

To confirm that the colonies growing on the culture medium (Figure 3) contain the target gene segment, PCR will be conducted using the primer pair pJET1.2. The initial size of the target gene segment was 214 bp. While the amplified plasmid DNA will measure 332 bp (which includes 118 bp from the pJET1.2 vector). The electrophoresis results (Figure 4) indicated that the vector containing the target gene segment has been successfully transformed into the bacteria.

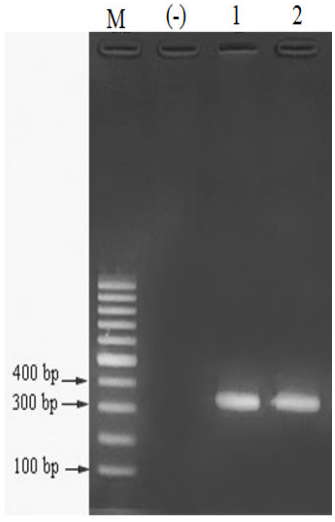

Figure 4. Electrophoresis result of PCR colonies with primer pair pJET1.2 (M: DNA ladder, (-): negative control, well 1: colony sample).

The sequencing results of the plasmid DNA sample, after successful cloning, show a 100% similarity with the gene sequence prior to insertion into the vector, and a 99.07% similarity with the ZYMV virus sequence (accession number DQ 925448.1) published in GenBank (Ha et al., 2008). This confirms that the gene segment inserted into the plasmid corresponds to the ZYMV virus gene.

3.3. Realtime RT-PCR reaction results

3.3.1. Generation of a standard curve

The correlation coefficient R^2 of the plasmid DNA sample is 0.9969 (Figure 5), which is with the acceptable limit (R^2 0.99), indicating that standard curve has high linearity, this supports accurate dilution calculations and pipetting of the required volumes. The standard curve equation is given by y = -3.417x + 49.605. The PCR reaction efficiency (E%) is 96.18%, and the slope is -3.417, both of which fall within the acceptable range. The standard curve was constructed based on the Ct threshold cycle value, using five standard points corresponding to five diluted concentrations of plasmid DNA, with each concentration repeated three times to enhance accuracy (Table 2). All plasmid DNA samples with concentrations of 10^9, 10^8, 10^7, 10^6, and 10^5 copies/µL were successfully amplified as shown by the amplification chart (Figure 6) and melting curve (Figure 7).

Table 2. Ct value of the reaction that amplifies the sample containing the Zucchini yellow mosaic virus gene segment

Sample (copies/µL)	Log of copies number	Threshold cycle (Ct) (Mean ± SD)	Coefficient of Variation (%)
10^9	7	18.51 ± 0.15	0.83
10^8	6	21.77 ± 0.38	1.75
10^7	5	26.18 ± 0.12	0.46
10^6	4	29.81 ± 0.43	1.43
10^5	3	33.79 ± 0.55	1.69
(-)	0	_	_

"-": negative control, "_": not detected.

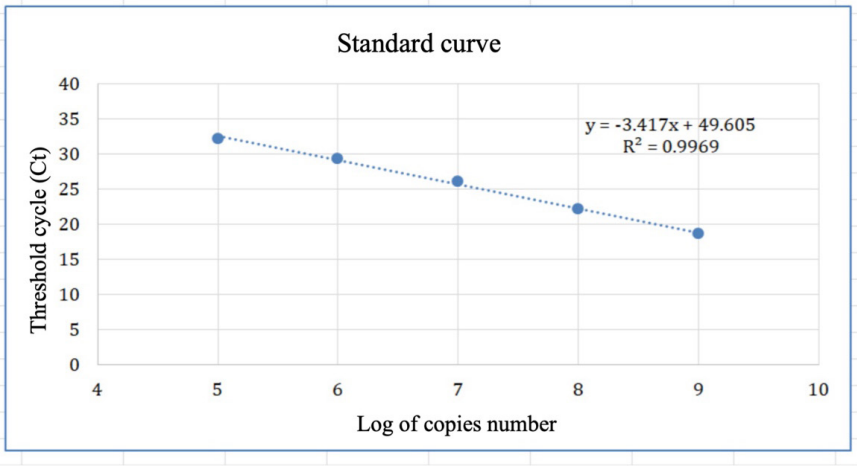

Figure 5. Standard curve generated from the plasmid containing the target gene.

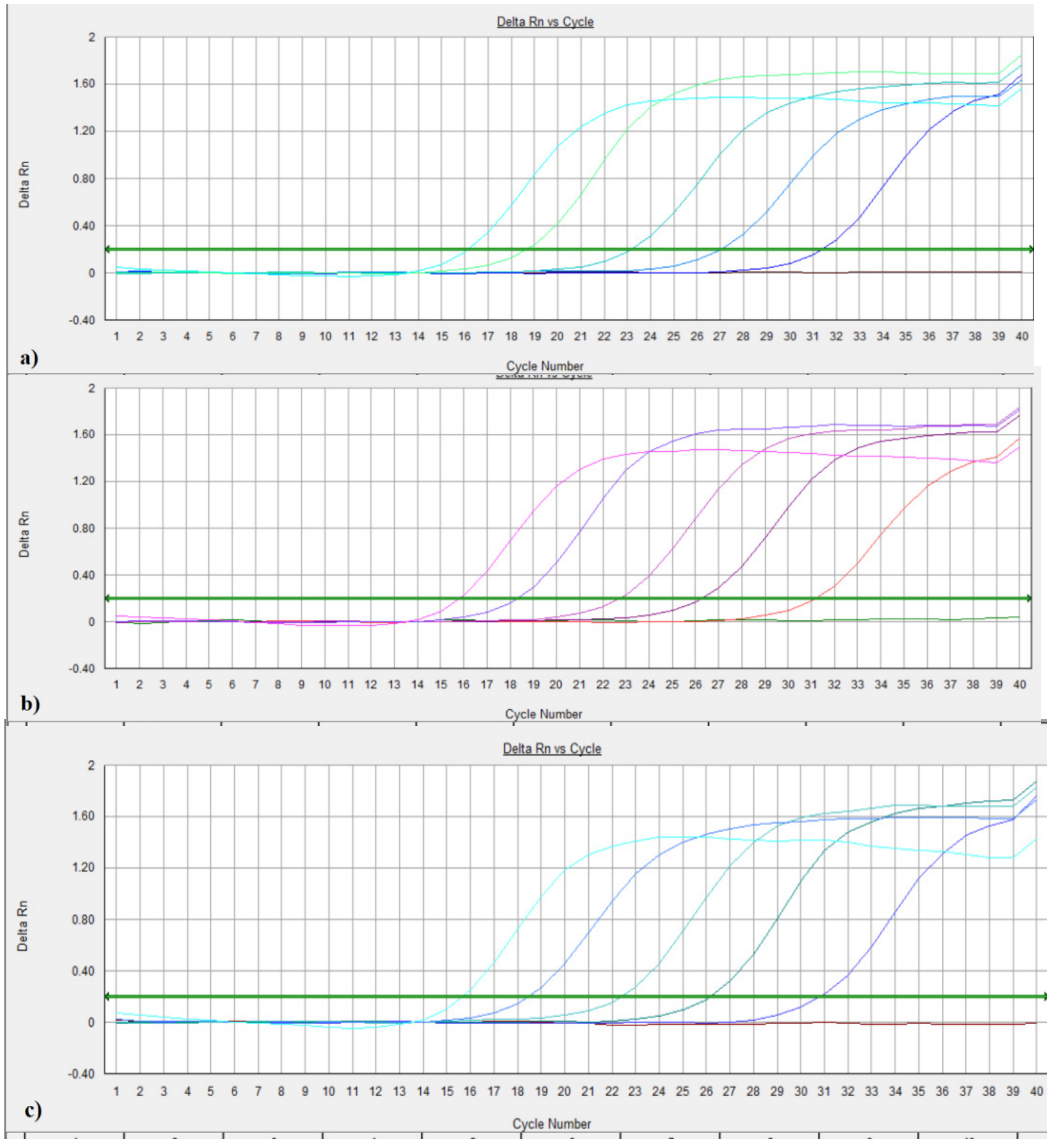

Figure 6. Amplification chart of Zucchini yellow mosaic virus gene segment based on fluorescence signal. (a): replication 1, (b): replication 2, (c): replication 3.

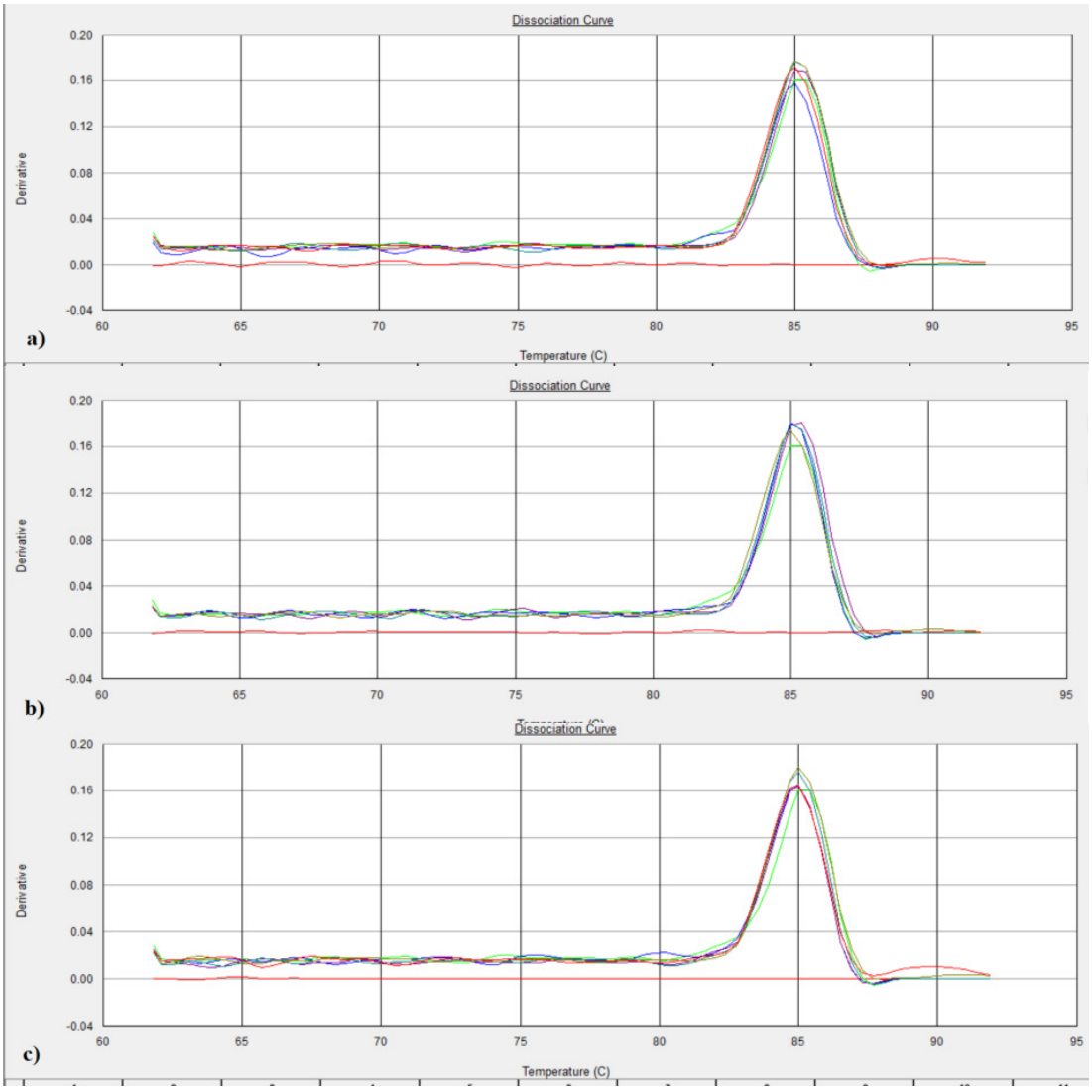

Figure 7. Melting curve diagram of Zucchini yellow mosaic virus gene fragment. (a): replication 1, (b): replication 2, (c): replication 3.

3.3.2. Evaluation of the specificity of the realtime RT-PCR

Table 3. The results for the specificity evaluation of the realtime RT-PCR

Sample	Threshold cycle (Ct)	Quantitative value (number of copies/ µL)	Qualitative
Zucchini yellow mosaic virus	18.45	1.3 x 10^9	+
Dasheen mosaic virus	_	_	-
Papaya ringspot virus	_	_	-
Chilli veinal mottle virus	_	_	-

"+": positive, "-": negative, "_": not detected.

The result in Table 3 showed that the RT-PCR assay did not detect the virulence gene in the cDNA of DsMV, PRSV, and ChiVMV. This result suggested that the primer was specified with ZYMV.

3.3.3. Diagnosis of ZYMV on field samples with realtime RT-PCR procedure

Of the 10 samples collected from garden 1, 4 were identified as positive for the ZYMV virus (Table 4). In garden 2, all 10 samples tested positive for the ZYMV virus. Notably, the quantification values for the 20 samples ranged from 7.1 x 10^6 to 8.5 x 10^9 copies/µL (Table 4).

Table 4. Threshold cycle (Ct) values of realtime RT-PCR for pumpkin leaf samples collected from the field

Garden	Sample	Realtime RT-PCR			PCR
		Ct	Quantitative value (Number of copies/µL)	Qualitative	Qualitative
	Negative control (-)	-	-	-	-
	Positive control (+)	18.45	1.3 x 10^9	+	+
Garden 1	1.1	25.48	11.5 x 10^6	+	+
	1.2	22.41	90.9 x 10^6	+	+
	1.3	16.14	6.2 x 10^9	+	+
	1.4	-	-	-	-
	1.5	-	-	-	-
	1.6	16.4	5.22 x 10^9	+	+
	1.7	-	-	-	-
	1.8	-	-	-	-
	1.9	-	-	-	-
	1.10	-	-	-	-
Garden 2	2.1	15.67	8.5 x 10^9	+	+
	2.2	26.14	7.4 x 10^6	+	+
	2.3	25.12	14.6 x 10^6	+	+
	2.4	25.44	11.8 x 10^6	+	+
	2.5	16.55	4.7 x 10^9	+	+
	2.6	23.77	36.4 x 10^6	+	+
	2.7	22.73	73.3 x 10^6	+	+
	2.8	23.17	54.5 x 10^6	+	+
	2.9	26.19	7.1 x 10^6	+	+
	2.10	24.93	16.6 x 10^6	+	+

(+): positive, (-): negative.

The qualitative results showed 100% concordance between the realtime RT-PCR method and the RT-PCR method. The results showed that the presence of ZYMV virus is quite common, causing losses in productivity and product quality during harvest, but to date there are no detailed statistics on losses caused by the disease. For the conventional RT-PCR technique, the PCR product needs to be electrophoresed to check the results, but with realtime RT-PCR technique, the amplification and quantification reactions take place at the same time, without the need for electrophoresis, which saves time and shortens the experimental steps compared to conventional RT-PCR technique. In addition, with high sensitivity, realtime RT-PCR technique can detect ZYMV virus early in the seed source or in seedlings before symptoms appear, thereby helping to eliminate pathogens early, reduce the possibility of spreading, save time, planting costs and care efforts.

4. Conclusions

The realtime RT-PCR technique demonstrated high specificity and accuracy in detecting the ZYMV virus. This diagnostic procedure was effective in quantifying the concentration of ZYMV in field-collected samples, revealing viral loads ranging from 7.1 x 10^6 to 8.5 x 10^9 copies/µL.

Conflict of interest

The author declares that there are no conflict of interest to disclose related to this manuscript.

Acknowledgements

This research was supported by the Research Institute for Biotechnology and Environment, Ho Chi Minh city Nong Lam University.

References

Aguiar, R. W. S., Martins, A. R., Nascimento, V. L., Capone, A., Melo Costa, L. T., Campos, F. S., Fidelis, R. R., Santos, G. R., Resende, R. O., & Nagata, T. (2019). Multiplex RT-PCR identification of five viruses associated with the watermelon crops in the Brazilian Cerrado. *African Journal of Microbiolog Research* 13(3), 60-69. https://doi.org/10.5897/AJMR2018.8976.

Ha, C., Revill P., Harding, R. M., Vu, M., & Dale, J. L. (2008). Identification and sequence analysis of potyviruses infecting crops in Vietnam. *Archives of Virology* 153(1), 45-60. https://doi.org/10.1007/s00705-007-1067-1.

Hu, J. S., Ferreira, S., Wang, M., & Xu, M. Q. (1993). Detection of cymbidium mosaic virus, odontoglossum ringspot virus, tomato spotted wilt virus, and potyviruses infecting orchids in Hawaii. *Plant Disease* 77(5), 464-468.

Lim, T. K. (2012). Cucurbita moschata. In Lim, T. K. (Ed.). *Edible medicinal and non-medicinal plants - Volume 2: Fruit*. Dordrecht, Netherlands: Springer. https://doi.org/10.1007/978-94-007-1764-0_41.

Moradi, Z., Mehrvar, M., & Nazifi, E. (2019). Population genetic analysis of Zucchini yellow mosaic virus based on the CI gene sequence. *Journal of Cell and Molecular Research* 10(2), 76-89. https://doi.org/10.22067/jcmr.v10i2.76133.

Sambrook, J., & Russell, D.W. (2001) *Molecular cloning: A laboratory manual* (3rd ed.). New York, USA: Cold Spring Harbor Laboratory Press.

Staroscik, A. (2004). *Calculator for determining the number of copies of a template*. Retrieved March 14, 2023, from http://cels.uri.edu/gsc/cndna.html.