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MOLECULAR DETECTION OF ZUCCHINI YELLOW MOSAIC VIRUS (ZYMV) INFECTING CUCURBITS IN IRAQ USING NESTED RT-PCR

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Abstract:
This study was initiated to investigate Zucchini yellow mosaic virus (ZYMV) using Reverse transcription polymerase chain reaction (RT-PCR) and species specific nested primers. Leaf samples were collected from symptomatic cucurbits grown in fields located at Al-Qadisiyyah and Baghdad provinces. Potyviruses were screened using double antibody sandwich enzyme linked immuno-sorbent assay (DAS-ELISA) and group specific primers. Nested RT-PCR was performed to confirm ZYMV in potyvirus positive samples. DAS-ELISA screening could detect potyvirus in 17 out of 181 cucurbit samples. Based on nested RT-PCR, 6 out of 17 were ZYMV infected. Sequence comparison confirmed the detection of ZYMV when DNA fragments amplified shared 99.29% maximum nucleotide identity with the equivalent ZYMV sequences from Iran (KU528623.1) and Israel (OL311706.1) indicating common origin. Neighbor-joining phylogenetic tree confirmed the relatedness when grouped all sequences isolated form zucchini squash, bitter apple, watermelon. melon and cucumber to equivalent ZYMV sequences from the Genbank. This study confirmed bitter apple is a new host to ZYMV. Thus, ZYMV can be a serious threat to cucurbits in Iraq as it showed to infects wild local hosts.
Keywords: Mosaic, wild gourd, potyviruses, nested primers, DAS-ELISA

Introduction

Zucchini yellow mosaic virus (ZYMV) is one of the most devastating viruses impacting cucurbits worldwide (Coutts et al., 2011). It belongs to the genus Potyvirus within the family Potyviridae (ICTV, 2022). ZYMV is transmitted horizontally by plant contact, aphids and vertically through pollen grains and seeds (Harth et al. 2017). Typical symptoms caused by ZYMV on cucurbits, including leaf mosaic, blistering and reduced growth and stunting resulting in poor fruit quality (Komata et al., 2014). ZYMV was reported for the first time in Italy on zucchini squash, 1981 (Lisa and Lecoq 1984). Then this virus was disseminated to many geographical locations causing several epidemics (Lecoq and Desbiez, 2012). In Iraq, ZYMV was reported to damage many cucurbits mainly, zucchini squash since 1990s causing serious losses up to 100% (Al-Ani et al 2011). Many studies were conducted to investigate ZYMV in Iraq, but they were restricted to serological and biological approaches (Adhab et al 2021; Merzah and Jarjees 2016; Jarjees and Merzah 2014). However, limited studies confirmed the presence of ZYMV based on molecular approaches (Al-Kuwaiti et al 2016, Alinizi, et al. 2021). This study, therefore, was initiated to investigate ZYMV in cucurbit samples using serological and molecular approaches.

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Materials and methods

Leaf samples (n=181) from symptomatic plants (Fig.1) grown in cucurbit fields in Al-Qadisiyyah and Baghdad provinces. Indirect DAS-ELISA was performed to investigate samples for virus incidence using monoclonal antibodies specific to potyviruses (Agdia, USA) following the manufacturer`s standard protocol. ELISA reader (Biotek, USA) was used to estimate potyvirus concentration at absorbance 405 nm. Total RNA was extracted from samples showed to be potyviraus infected by AccuZolTM Total RNA commercial kits (Bioneer, South Korea), quantified by Nano-drop spectrophotometry and used for cDNA synthesis using cDNA RT-Premix commercial kits and random hexamers (Bioneer, South Korea) following the manufacturer`s instructions. Nested primer sets designed were designed using Primer3 plus software (Table 1). Nested-PCR was performed using GoTaq ®Green PCR Master Mix (Promega, USA) according to protocol recommended by the manufacturer. First round nested- PCR was set up using program listed in tables (2). Similar steps were followed for the 2nd round except for annealing temperature which was 58 ºC. Nested-PCR products were analyzed using ethidium bromide gel electrophoresis (Sambrook and Russell, 2006). Nested-PCR products were sent for sequencing (Macrogen, South Korea), Sequence analysis was performed using MEGA 6 software (Tamura et al., 2013).

Table 1 Nested primer sets used in this study to detect ZYMV in cucurbit samples

Primer	Sequence 5'-3'		Amplicon	Targeted gene	Location
ZYMV-PCR External primers	F	TCCGGCTCAGGTGAGAAAAC	611bp	Polyprotien/gp1	139-9381
	R	GCTTTCATCTGCGCTACAGC			
ZYMV-Nested PCR Internal primers	F	CCGCGTCTTTCGAAGATCAC	419bp	Polyprotien/gp1	139-9381
	R	TACCTCGGCATGTATGGTGC			

Table 2 Nested PCR protocol

Step	Time/Temperature	Cycle
Pre-denaturation	95 °C 5 min.	**1**
Denaturation	95 °C 30 sec.	**35**
Annealing	59 °C 30 sec.	
Extension	72 °C 1min.	

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Final extension	72 °C 5 min.	**1**
Hold	4 °C	**ω**

Results and discussion

Indirect DAS-ELISA could decoct potyvirus infection in 17 out of 181 cucurbit samples tested when scored the highest absorbance values, at 405 nm, ranged 2.578-3.363 compared to 0.385 and 3.466 for negative and positive controls, respectively (Table 3). When tested by nested PCR, 6 out of 17 samples showed to be ZYMV infected when nested primers amplified the DNA fragments of the expected size, ~419 bp (Fig. 2 and table 3).

Table 3: Cucurbit samples detected to be ZYMV infected

No.	Sample	Host	ELISA	Acc. code	Location	Collection date	Symptoms
1	Z1	*Cucurbita pepo*	2.939	OP730940	Al-Jadriya/Baghdad 33.171748°N 44233481°E	202/11/91	R, L, M, F (Fig1 a)
2	Z2	*Citrullus colocynthis*	3.163	OP730941	Afak/Al-Qadisiyyah 32.0345°N 45.1434°E	2021/9/1	V, M (Fig1 b)
3	Z3	*C. lanatus*	2.888	OP730942	Afak/Al-Qadisiyyah 32.0345°N 45.1434°E	2021/9/5	M, L, R, D (Fig1 c)
4	Z4	*Cucumis melo*	2.945	OP730943	Afak/Al-Qadisiyyah 32.0345°N 45.1434°E	202/6/172	M, R, Y (Fig1 d)
5	Z5	*C. sativus*	3.363	OP730944	Yusufiyah/Baghdad 33.430°N 44.1442°E	202/4/302	M, C, Y (Fig1 e)

D: dwarfing, C: leaf curling, F: fruit malformation, L: leaf malformation, M: mosaic, R: reduction of leaf area, V: vein clearing, Y: yellowing

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Figure 1: Symptoms associated with ZYMV naturally infected cucurbits, a. zucchini, b. bitter apple. C, watermelon, d. melon and e cucumber.

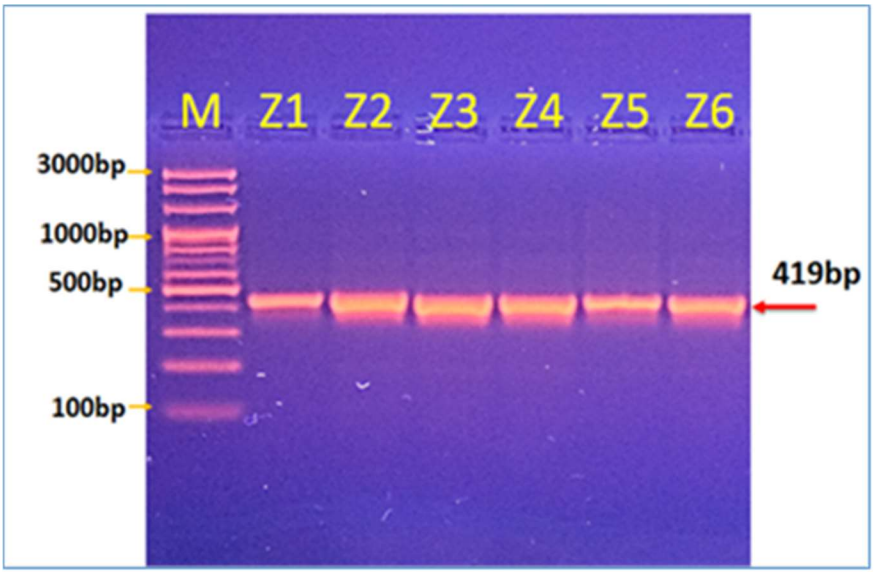

Figure 2: Ethidium bromide gel electrophoresis showing ~419 bp DNA fragments amplified by nested primers specific for ZYMV. Z1-Z6: cucurbit samples, M: 100 bp DNA marker (Bioneer, South Korea).

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Sequence comparison confirmed DNA fragment amplified shared maximum 99.29 % identities to equivalent ZYMV sequences from Iran (KU528623.1) and Israel (OL311706.1) indicating common origin. Neighbor-joining phylogenetic tree grouped all sequences obtained to those retrieved from NCBI gene bank confirming they are closely related to ZYMV (Fig. 3). Sequence analysis suggests nested-primer sets designed could detect ZYMV in infected sample, successfully. Thus, nested RT-PCR approach can be used to screen cucurbit samples for ZYMV infection. ZYMV infection was confirmed in 2 Citrullus species including bitter apple C. colocynthis. This genus was reported as a source of inherited resistance against ZYMV isolates from the USA (Guner et al., 2019). However, Iranian isolate showed to infect both watermelon and bitter apple (Massumi et al., 2011). The high nucleotide sequence identity and biological data suggest ZYMV may have been introduced from Iran in the near past, but not from USA (Alinizi, et al. 2021). ZYMV isolates can be a serious threat to cucurbits in Iraq as it showed to infect hosts reported to be ZYMV resistant. The use of resistance varieties may be insufficient to control ZYMV in Iraq. Recently, grifted verities using (Cucurbita maxima x Cucurbita moschata) stocks were widely used in Iraq to improve production, drought, salinity and root disease resistance (Abdul Razzaq 2017). This agricultural practice may increase the risk of new resistance breaking variants or recombinant strains emergence (Heo et al., 2020). Rapid action and precaution procedures are required to protect zucchini and other cucurbits against ZYMV in Iraq.

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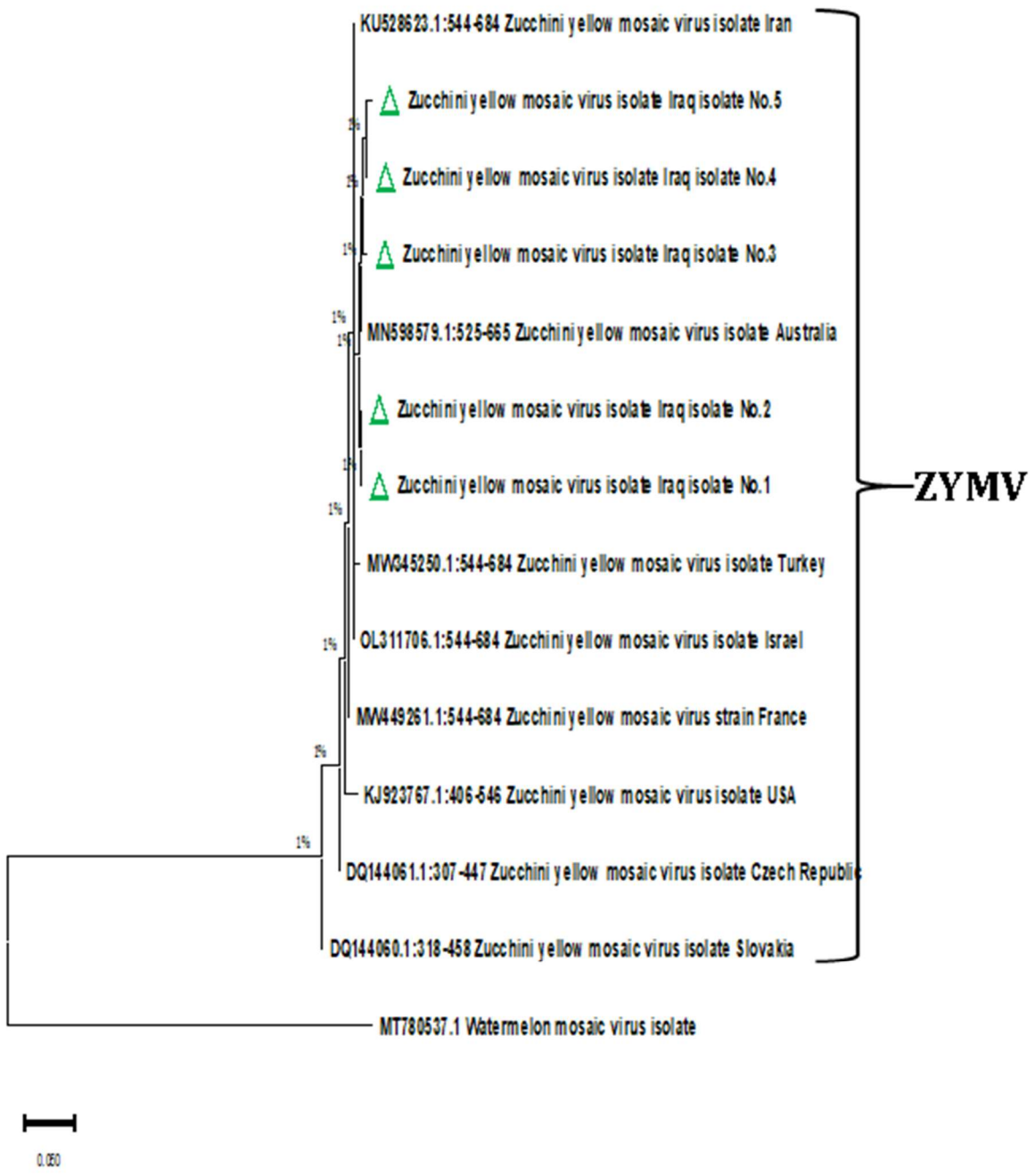

Figure 3: Neighbor-joining phylogenetic trees constructed from ZYMV partial nucleotide sequences of Iraqi isolates (marked with Δ) and equivalent sequences from the Genebank. Watermelon mosaic virus (WMV) was included as an outgroup comparison.
Isolates No1: zucchini, No2: bitter apple, No3: watermelon, No4: melon and No5: cucumber.

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