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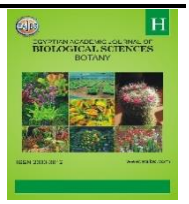

Detection of Zucchini Yellow Mosaic Virus-Infected Squash Plants in Egypt

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ABSTRACT

Background: The Zucchini yellow mosaic virus (ZYMV) is well recognized as a very significant viral pathogen that causes substantial yield reductions in cucurbitaceous crops on a global scale. **Methods:** To detect and identify ZYMV in squash plants we used the serology reaction, transmission electron microscope, and molecular methods. **Results:** ZYMV was first isolated from naturally infected squash plants in the Alexandria governorate of Egypt, where it caused mosaic, yellowing, and deformation of the leaves. In addition to a molecular diagnosis utilizing RT-PCR, the identification relied on serological responses with antisera to CMV, SqMV, and ZYMV. Using centrifugation and PEG, the virus was cleaned. The A260/280 and A280/260 ratios of the purified virus were 1.38 and 0.72, respectively, which are consistent with those of a nucleoprotein. 2.36 mg of pure virus was recovered from per 100g of infected leaf material. The virus particles, as seen under an electron microscope after a partial purification preparation technique, have a flexuous filamentous structure and measure around 750 nm in length and 12 nm in diameter. Finally, the molecular investigation established the identification of the ZYMV isolate after a specific antiserum with a titer of $1:2.56\times10^4$ was generated and tested at the third week.

INTRODUCTION

One of Egypt's most extensively cultivated crops is squash (*Cucurbita pepo* L.), which can be cultivated year-round in the country's many greenhouses and tunnels. Carbohydrates, soluble fiber, pro-vitamin A, and protein may all be found in squash, which is why it is cultivated for human consumption. About half of all plant diseases are attributed to viruses, and agriculture plays a significant role in their dissemination (Bernardo *et al*., 2018). More than 700 plant species are susceptible to infection by viruses (Chauhan *et al*., 2019). The potyvirus family includes the virus responsible for the widespread devastation of cucurbit crops known as zucchini yellow mosaic virus (ZYMV). In addition to causing output losses of up to 94% (Tohamy *et al*., 2018), plant yellowing and stunting caused by ZYMV can lead to severe leaf and fruit abnormalities. In 1981, researchers in northern Italy (Lisa *et al*.,1981) isolated the virus, and in 1983, researchers in Egypt (Provvidenti *et al*., 1984) characterized it for the first time. This agricultural pathogen has significant economic implications. Controlling this catastrophic crop disease requires an in-depth knowledge of

ZYMV's epidemiology and evolutionary history (Simmons *et al*., 2011). This virus is spread by contact with plants, seeds, and mechanically (Tymchyshyn *et al*., 2017). The virus has been proven to be transmitted by a significant number of aphid species, although the greatest reported transmission efficiency has been found in two species: *Myzus persicae* and *Aphis gossypii* 41% and 35%, respectively (El-Borollosy, 2015). A positive-sense ssRNA and a 5' genome-linked protein (VPg) make up the monopartite genome of ZYMV particles (Anthony-Johnson et al., 2013). The particles are 750 nm in length and are curved. Many potyvirus proteins have more than one purpose, with the P3 proteins being particularly variable throughout potyvirus genomes and hence being viewed as important virulence factors (Tymchyshyn *et al*., 2017). Both serological and nucleic acid-based approaches employing polymerase chain reaction (PCR) were utilized to diagnose ZYMV in the lab (Amer, 2015; Ghanem *et al*., 2016).

This investigation set out to determine the causative agent of ZYMV using symptoms, serology, RT-PCR, photometrical characteristics of the pure virus, and electron microscopy of the viral particle. Additionally, antiserum was produced in response to the isolated virus.

MATERIALS AND METHODS

Symptoms and Inoculum Preparation:

Leaf samples were taken from squash plants cultivated in Borg-Alarb region, the Alexandria Governorate, Egypt, that showed signs of severe mosaic, chlorosis, and deformation of the leaves. After dusting the leaves of the plants, the inoculum was made by crushing infected leaf tissue in 0.1 M phosphate buffer (PB). The inoculum was then applied to the leaves of the plants using the fingers. The isolated virus was kept alive on squash plants which provided the virus's source for further research.

Identification Based on Serological Reaction:

The experiment employed antisera targeting cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV), and squash mosaic virus (SqMV). The antisera for CMV and ZYMV were developed internally at our lab, located in Alexandria, Egypt. The antiserum for SqMV was obtained from AGERI, which is affiliated with the Agricultural Research Center (ARC) in Egypt.

The methodology for the indirect ELISA was stated by (Abd El-Aziz and Younes, 2019). Using a mortar and pestle, the plant samples were crushed to a 1:10 (w/v) consistency in the coating buffer. The mixture was then strained through double-layered cheesecloth. Antigens were added to the bottom of the wells by adding 100 µl of each sample, and the wells were then incubated for three hours at 37°C. By filling wells with PB-Saline Tween (PBST) for five minutes, the plates were washed three times. Antisera were diluted 1:400 for ZYMV and CMV and 1:250 for SqMV with filtered extract from healthy tissue at a ratio of 1:20 (w/v) in PBST containing 0.2% BSA and 2% soluble polyvinyl pyrrolidone (PVP)) to reduce non-specific reactions. The samples were then incubated for 45 minutes at 37 °C. Centrifugation was used to remove the precipitate, which was generated, for 10 minutes at 5000 rpm. Each well received 100 µl aliquots of the diluted antisera. The plates were then incubated for two hours at 37 °C, and then they were cleaned as usual.

Each well had 100 µl of a 1:1000 serum buffer dilution of goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (Sigma Aldrich, USA) added to it, incubated at 37 °C for an hour, and then washed. Each well was supplemented with 100 µl of 10% diethanolamine buffer, pH 9.8, containing 1mg/ml para-nitrophenyl phosphate, and then incubated at room temperature for 30 minutes to allow the enzyme to activate its substrate. 50 µl of 3M NaOH was added to cease the enzyme reaction. The absorbance at 405 nm was used to represent the ELISA data obtained using a Sunrise ELISA reader. Absorbance

readings that are at least twice as high as the healthy control are regarded favorably. Antigen-free wells (coating buffer alone) were used in the experiment as a control.

Identification Based on RT-PCR:

Following the protocol outlined by the manufacturer (Bioline cat No.Bio-52040), viral RNA was isolated from the purified virus using the (plant RNA Mini Kit). Using forward (5'-GGACAGTGCGACTATAGCTTCAA -3') and reverse (5'-TTTAACCGCGAATTGCGTATC-3') primers from the ZYMV-coat protein (CP) gene, we generated first-strand cDNA and amplified it by polymerase chain reaction (PCR). PCR process was initially denaturized at 95 °C for 2 min, followed by 35 cycles at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min. The last ten minutes of the extension were carried out at 72 °C. From 1 ng of RNA, cDNA was synthesized with the use of a Maxima Reverse Transcriptase kit. Following the protocol published by Heflish *et al*. (2021), PCR reactions were performed using 2 µL of produced cDNA and gene-specific primers for ZYMV-CP. Gel electrophoresis on 1% agarose was used to separate the PCR products before being stained with red safe and evaluated using a gel documentation system.

Purification of ZYMV and TEM Microscopy:

The isolated ZYMV was purified in a manner like that reported by Younes (2003), with a few tweaks. Three weeks after inoculation, 100 g of freshly harvested, systemically infected squash leaves were blended with 300 ml of extraction buffer pH 8.5. Three layers of cheesecloth were used to filter the homogenate before 3% Triton X-100 (v/v) was added. After 30 minutes of stirring at room temperature, the homogenate was centrifuged at low speed (12000 rpm/15 min) at 4 °C after being treated with a combination of chloroform and carbon tetrachloride (1:1, v/v). After stirring the aqueous phase for an hour at 4°C with 3% NaCl (w/v) and 8% PEG, using a Beckman centrifuge, the mixture was centrifuged at 12000 rpm for 20 minutes at 4°C. Overnight at 4°C with intermittent stirring, the pellets were kept in a solution of 0.05 M sodium citrate buffer (SCB), pH 7.5. The pellet was resuspended and then centrifuged at 4 °C for 20 minutes at 12000 rpm. High-speed centrifugation (23,500rpm/2h) was used to separate the supernatant. The pellet is suspended in a 0.05M SCB and centrifuged at (9000rpm/20min). The concentration of the virus was calculated using an extinction coefficient. Drops of pure viral preparations floated for 5 minutes on formvar-coated nickel grids, which were used for Transmission electron microscopy (TEM) (JEM-1400—Jeol Ltd., Tokyo, Japan).

Production of ZYMV Antiserum:

Antiserum to ZYMV was developed in white rabbits by injecting them intramuscularly with pure virus over the course of three weeks. For the initial injection, the virus at a concentration of 0.24 mg/ml was emulsified with an equivalent amount of Freund's complete adjuvant, and for the rest injections using the incomplete. Three weeks following the fourth injection, the rabbit bled. The collected antiserum for ZYMV was kept at -20 °C with 0.05% sodium azide.

Indirect ELISA was used, as was previously reported, to measure the titer of ZYMV antiserum. A coating buffer was used to dilute extracts from infected and healthy squash plants to a 1:10 ratio. Antiserum was prepared by cross-adsorption using filtered extracts of healthy tissues diluted 1:20 in serum buffer, and then serially diluted up to 1:256,000.

RESULTS AND DISCUSSION

Symptoms of the Isolated Virus:

Based on the symptoms alone, ZYMV was originally detected in leaf samples from squash plants exhibiting severe mosaic, chlorosis, and leaf deformation (Figure 1). Infection with ZYMV has been reported on cucurbit crops all over the globe, and similar clusters of

symptoms have been recorded earlier (Müller *et al*., 2006; El-Hoseny *et al*., 2010; Wang and Li, 2017).

Fig. 1. Infected squash leaves showing severe mosaic, chlorosis, and deformation.

Serological Reaction:

Tests with antibodies showed that ZYMV antiserum diluted 1:400 in indirect ELISA worked against the virus that was isolated. According to results from indirect ELISA, neither CMV nor SqV antisera elicited a positive response. Ghanem (2003), who studied the Okra leaf curl virus, and Mostafa and Abou-Ela (2011), who studied ZYMV, came to similar conclusions.

Table 1. The response of a squash leaf extract infected with viruses, namely CMV, SqMV, and ZYMV, was assessed using an indirect enzyme-linked immunosorbent assay (ELISA) using antisera.

Squash leaf extract	**ELISA reading**		
	CMV	SqMV	ZYMV
Infected	0.33	0.20	2.81
Healthy	0.44	0.21	0.40

Molecular Detection:

The findings of Hosseini *et al*. (2007) and Zheng *et al*. (2010) who effectively identified ZYMV by RT-PCR, are comparable with the amplification results of 458 bp of the RNA recovered from squash infected with ZYMV (Figure 2).

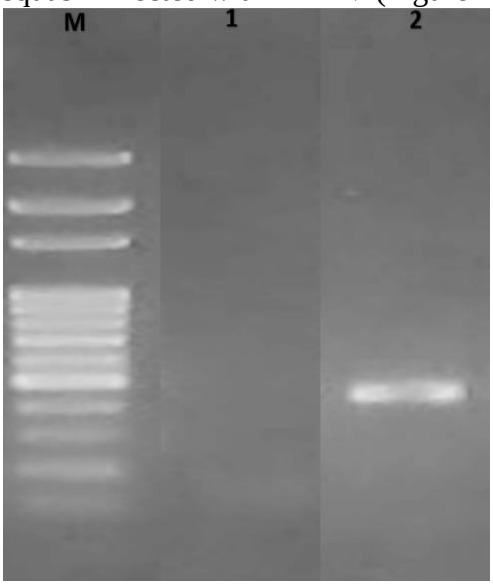

Fig. 2. ZYMV coat protein RT-PCR electrophoresed pattern. M = 100 bp ladder, 1= healthy control, 2= infected

Characteristics and Virus Yield:

ZYMV's squash isolate was refined using PEG and centrifugation. The pure viral isolate's absorption spectra, as evaluated are characteristic of nucleoprotein absorption (Figure 3A). The pure viral preparation's UV absorption spectra showed a typical nucleoprotein spectrum. Uncorrected for light scattering, the ratios of A260/280 and A280/260 were 1.38 and 0.72, respectively. About 2.36 mg of pure virus were produced for every 100 grams of fresh squash leaves. According to a transmission electron microscopy (TEM) analysis, the refined ZYMV particles had a size of around 750 nm and were flexuous filamentous (Figure 3B). These findings are supported by those reported by Wang and Li (2017), Khalifa *et al.* (2015), and Hosseini *et al*. (2007).

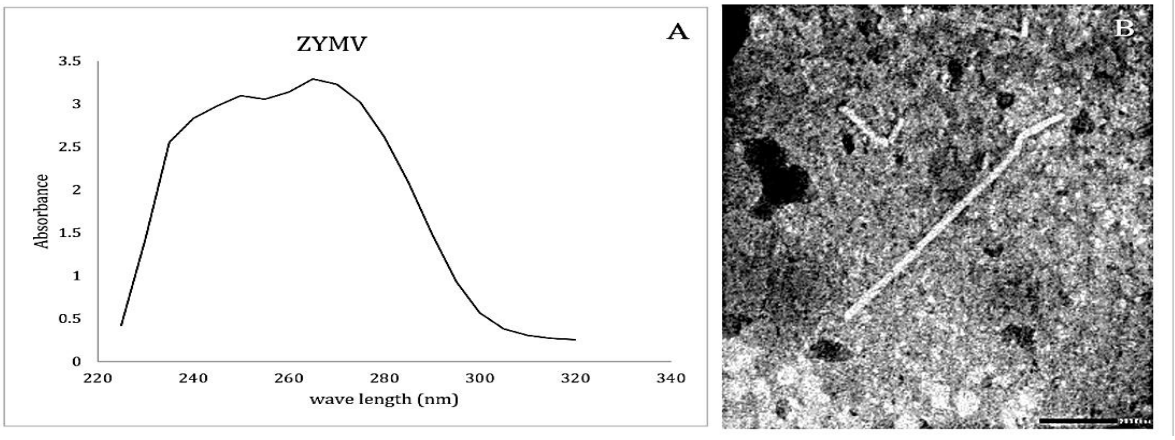

Fig. 3. Purified ZYMV spectrum kinetics (A). Electron micrographs showing flexuous filamentous particles, measuring 750 × 12 nm (B).

Production of ZYMV Antiserum:

A ZYMV-specific antiserum was generated. The determination of antiserum titer was conducted using the ELISA method. Table 2 demonstrates that ELISA readings were seen to be positive up to dilutions of $1:2.56\times10^4$ in the third week.

Table 2. The ZYMV-infected plant extract's ELISA readings titer at different collecting weeks.

Antiserum dilution	First week		Second week		Third week	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
$1:4\times10^2$	0.362	1.338	0.348	1.442	0.556	2.071
$1:8\times10^2$	0.315	1.028	0.262	1.256	0.494	1.440
$1:1.6\times10^3$	0.133	0.725	0.080	0.989	0.277	1.375
$1:3.2\times10^3$	0.029	0.383	0.001	0.589	0.062	0.919
$1:6.4\times10^3$	**0.001**	**0.141**	0.001	0.318	0.001	0.696
$1:1.28\times10^4$	0.001	0.001	**0.001**	**0.145**	0.001	0.446
$1:2.56\times10^4$	0.001	0.001	0.001	0.001	**0.001**	**0.262**

The results were done twice, and the ELISA values at 405 nm are an average of two repetitions each. A positive reading value was one that was at least twice that of the healthy control.

Conclusions

In conclusion, our study shows that one of the major Cucurbitaceae crops cultivated in northern Egypt is squash. Stunting of the plants, yellow mosaic, and deformity of the leaves were noted during the field study. Squash seedlings' artificially generated symptoms and

their natural symptoms were identical. Positive results were obtained using indirect ELISA using ZYMV antiserum, and ZYMV was identified by RT-PCR with a 458 bp product size. ZYMV was isolated from infected squash leaves using PEG and centrifugation. The infected leaves produced a good yield. Indirect ELISA revealed a high titer.

REFERENCES

Abd El-Aziz, M. H., and Younes, H.A.2019. Detection of Cucumber mosaic cucumovirus in infected cowpea plants (*Vigna unguiculata* L.) from northern Egypt. *Novel Research in Microbiology Journal*, 3:2, 326-340.

Amer, M.A., 2015. Serological and molecular characterization of Cucurbit chlorotic yellows virus affecting cucumber plants in Egypt. *International Journal of Virology*, 11: 1, 1-11.

Anthony Johnson, A.M., Vidya, T., Papaiah, S., Srinivasulu, M., Mandal, B., and Sai Gopal, D.V.R., 2013. First report of Zucchini yellow mosaic virus infecting Gherkin (*Cucumis anguira*) in India. *Indian Journal of Virology*, 24: 289-290.

Bernardo, P., Charles-Dominique, T., Barakat, M., Ortet, P., Fernandez, E., Filloux, D., Hartnady, P., Rebelo, T.A., Cousins, S.R., Mesleard, F., and Cohez, D. 2018. Geometagenomics illuminates the impact of agriculture on the distribution and prevalence of plant viruses at the ecosystem scale. *The ISME journal*, 12: 1,173-184.

Chauhan, P., Singla, K., Rajbhar, M., Singh, A., Das, N., and Kumar, K., 2019. A systematic review of conventional and advanced approaches for the control of plant viruses. *Journal of Applied Biology and Biotechnology*, 7: 4, 89-98.

El-Borollosy, A.M., 2015. Aphid-transmission efficiency of Cucumber mosaic cucumovirus and Zucchini yellow mosaic potyvirus on squash and their control using essential plant oils. *Journal of Basic and Applied Scientific Research*, 5:1, 18-26.

El-Fallal, A.A., El-Sayed, A.K., Diab, A., and Sadik, A.S., 2015. Identification of an isolate of zucchini yellow mosaic potyvirus infecting squash in Egypt. *Pakistan Journal of Biotechnology*, 12: 2,173-180.

El-Hoseny, M.E., Amira, E.F.A., El-Sayed, A.K., Diab, A., and Sadik, A.S. 2010. Biology, cytopathology and molecular identification of an Egyptian isolate of Zucchini yellow mosaic potyvirus (ZYMV-Eg). *Pakistan Journal of Biotechnology*, 7:1-2, 75-80.

Ghanem, G.A.M. 2003. Okra leaf curl virus: A monopartite begomovirus infecting okra crop in Saudi Arabia. *Arab Journal of Biotechnology*, 6:1, 139-152.

Ghanem, G.A.M., Noura-Hassan, M., Kheder, A.A., Mazyad, H.M., and Abdel-Alim, A.I. 2016. Antiserum production, biological and serological detection of Cucurbit yellow stunting disorder crinivirus (CYSDV) in Egypt. *International Journal of Advanced Research*, 4:4, 1116-1128.

Ghobary, H.M.M. and Ibrahim, K.Y., 2010. Improvement of summer squash through inbreeding and visual selection. *Journal of Agricultural Research Kafer El-Sheikh Univ*, 36: 3, 340-350.

Heflish, A.A., Abdelkhalek, A., Al-Askar, A.A., and Behiry, S.I., 2021. Protective and curative effects of *Trichoderma asperelloides* Ta41 on tomato root rot caused by *Rhizoctonia solani* Rs33. *Agronomy*, 11: 6, 1162.

Hosseini, S., Mosahebi, G.H., Koohi Habibi, M., and Okhovvat, S.M., 2007. Characterization of the Zucchini yellow mosaic virus from squash in Tehran province. *Journal of Agricultural Science and Technology*, 9: 2, 137-143.

Hosseini, S., Mosahebi, G.H., Koohi Habibi, M., and Okhovvat, S.M. 2007. Characterization of the Zucchini yellow mosaic virus from squash in Tehran Province. *Journal of Agricultural Science and Technology*, 9: 137-143.

Lisa, V., Boccardo, G., D'Agostino, G., Dellavalle, G., and d'Aquilio, M. 1981. Characterization of a potyvirus that causes zucchini yellow mosaic. *Phytopathology*, 71: 7, 667-672.

Mady, M.A., 2009. Effect of some phosphorus compounds as seed-soaking materials on winter squash (*Cucurbita pepo* L.) plants. *Journal of Plant Production*, 34: 6, 6727-6739.

Mostafa, F.A.M. and Abou-Ela, A.A. 2011. Sensitive detection of watermelon mosaic and zucchini yellow mosaic viruses from infected squash plants using serological methods and polymerase chain reaction. *The Egyptian Journal of Experimental Biology* (*Botany*), 7: 179-185.

Müller, C., Bröther, H., Von Bargen, S., and Büttner, C. 2006. Zucchini yellow mosaic virus incidence and sources of virus infection in field-grown cucumbers and pumpkins in the Spreewald, Germany. *Journal of Plant Diseases and Protection*, 113: 6, 252–258

Provvidenti, R., Munger, H.M., and Paulus, H.O., 1984. Epidemics of Zucchini yellow mosaic virus and other cucurbit viruses in Egypt in the spring of 1983. *Cucurbit Genetics Cooperative*, 33.

Simmons, H.E., Holmes, E.C., Gildow, F.E., Bothe-Goralczyk, M.A., and Stephenson, A.G. 2011. Experimental verification of seed transmission of Zucchini yellow mosaic virus. *Plant Disease*, 95: 751-754.

Tohamy, M.R., Kheder, A.A., Ghanem, G.A.M., and Sulaiman, T.M., 2018. Biological, serological and molecular characterization of Egyptian zucchini yellow mosaic virus isolate infecting squash plants in Fayoum governorate. *Zagazig Journal of Agricultural Research*, 45: 6, 2377-2388.

Tymchyshyn, O., Shevchenko, T., Shevchenko, O., and Budzanivska I. 2017. Phylogenetic analysis of seed-transmitted isolate of Zucchini yellow mosaic virus. *БІОЛОГІЯ.* 2: 74, 47-50.

Wang, D. and Li, G. 2017. Biological and molecular characterization of Zucchini yellow mosaic virus isolates infecting melon in Xinjiang, China. *Canadian Journal of Plant Pathology*, 39: 1, 48-59.

Younes, H.A. 2003. Natural infection of luffa (*Luffa aegyptiaca* Mill) with zucchini yellow mosaic virus in Egypt. *Journal of the Advances in Agricultural Researches*, 8:227-239.

Zheng, L., Rodoni, B.C., Gibbs, M.J., and Gibbs, A.J. 2010. A novel pair of universal primers for the detection of potyviruses. *Plant Pathology*, 59: 2, 211-220.