Biological and Molecular Identification of Severe *Zucchini Yellow Mosaic Virus* from *Cucurbita pepo* cv. Eskandarani

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ABSTRACT

Zucchini yellow mosaic virus (ZYMV) was isolated from *Cucurbita pepo* cv. Eskandarani cultivated in the open field showing severe systemic symptoms. The isolated virus has wide host range belonging to 4 families resulting systemic and local symptoms. Stability of the isolated virus *in vitro* was found as 55 ^{o}C for TIP, 10^{-4} for DEP and LIV 4 days. The characteristic spectrophotometric data of the highly purified virus showing that maximum and minimum absorbance was 250 and 260 nm respectively; the ratio for A_{280}/A_{260} was 1.004; and the $A_{max.}/A_{min.}$ was 1.099 with the final yield as 0.883 mg/ml. Electron microscopy showed the filamentous flexuous particles with about 750 X 12-15 nm. The virus was identified as ZYMV by RT-PCR technique using high specific primers as 113 bp fragment within the nuclear inclusion protein b (NIb) coding region of the virus genome. The cytopathological effects of ZYMV on the infected cells were completely chloroplasts destruction, and deformation of vascular bundles. The effect of late ZYMV infection decreased fresh weight and yield of squash plants.

Key words: ZYMV, stability, RT-PCR, electron microscopy.

INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is a member of the genus *Potyvirus* of the family *Potyviridae*, it is the one of the most aggressive and destructive virus causes yield losses in cucurbits crops worldwide. The virus was first reported in Italy and France in 1981 (Lisa *et al.,* 1981) and subsequently from other countries. ZYMV characterized by a monoparticle, positive-sense, single stranded RNA genome encapsidated consists of about 9600 nucleotides (Balint *et al*., 1990) with a $^{/}5$ viral protein genome linked (VPG) and a poly (A) tail in flexuous rod shape particles (Dougherty and Semler, 1993).

Like other potyviruses, ZYMV is efficiently transmitted by aphids in a nonpersistent manner (Lisa *et al.,* 1981), however, the rate of virus transmission via seed is low and difficult to prove (Provvidenti and Robinson 1987). Control of ZYMV is difficult but the use of resistant cultivars, inoculation of mild ZYMV-WK strains for cross protection against severe challenging strains (Lecoq *et al.,* 1991 and Walkey *et al.*, 1992) and the use of mineral oil sprays (Makkouk and Menassa, 1986) in association with pyrethroids (Raccah *et al.*, 1985), might provide protection under certain ecological conditions. Fegla *et al.,* (2009) were successfully isolated ZYMV from naturally infected squash plants in Northern Egypt and it was infected representative of 4 plant families; and the purified virus particles suspension has U.V. spectra relative to $A_{260/280}$, $A_{280/260}$ and $A_{max/min}$ as 0.78, 1.96 and 1.29.

This paper aims to characterize one of the Potyviruses caused severe infection led

to big and significantly decreasing in squash (*C. pepo*) plantations, which is provisionally named *Zucchini yellow mosaic virus*.

MATERIALS AND METHODS

Source of the virus isolate:

The samples were collected randomly during growing season 2013 from open field-grown squash (*C. pepo* cv. Eskandarani) in Faculty of Agric. Ain Shams Univ., Cairo, Egypt on the stage of plant growth before the setting of the fruits. Natural collected infected samples external viral symptoms like such as: mottling, mosaic, yellowing, leaf distortion and reduction of yield (Fig. 2) were directly transferred in plastic pages and icebox to Virology Laboratory, Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt for the following studies.

Virus isolation and propagation:

The virus was isolated biologically by single local lesion method according to Noordam (1973). For biological purification, one single local lesion was separated and grinding in 1ml of 1:2 (w/v) of 0.1 M Phosphate buffer, pH 7.2, and then the extracted sap was mechanically inoculated on middle leaves of indicator plants *Chenopodium amaranticolor*. The extraction of resulted local lesions was inoculated on healthy *Cucurbita pepo* cv. Eskandrani plants as a ZYMV propagative host. The inoculated plants were kept in an insect proof under greenhouse conditions (25-28 ^{0}C) for 21 days.

Biological properties (Host Range)

Fifteen plant species belonging to 5 families were mechanically inoculated with the infectious virus sap and thin maintained in an insect-proof greenhouse to observe symptoms on both inoculated and non-inoculated leaves. The latent infection for external symptoms was conducted by back-inoculation to healthy *C. pepo* cv. Eskandrani.

In vitro properties of the isolated virus:

The *in vitro* properties of the isolated virus including thermal inactivation point (TIP), dilution end point (DEP) and longevity *in vitro* (LIV) was determined in infectious crude sap extracted from inoculated squash leaves after 4 weeks post-virus inoculation according to Walkey (1985) by using healthy *C. pepo* cv. Eskandrani as systemic host.

Virus purification:

The isolated virus particles were purified from 200 g infected squash tissues by the method of Azzam and Makkouk (1986), which it was based on low and high speed centrifugation, followed by sucrose density gradient centrifugation.

The purified virus suspension was evaluated spectroscopy ranged from 220-300 nm in Shimadzu UV-2401 PC UV-Vis recording spectrophotometer (Molecular Biology Lab. National Research Centre) and the virus yield was determined using extinction coefficient of ZYMV 2.4 (Huang *et al.*, 1989).

Electron microscopy:

Virus morphology:

Electron microscopic examination of negatively stained leaf-dip preparation by uranyl acetate was carried out according to Ahlawat and Varma (1997) and examined under a JOEL- JM-100-C transmission electron microscope (TEM) (Electron Microscope Unit, AL-Azhar University, Cairo, Egypt).

Ultrathin section:

Several blade tissues from top leaves of the infected squash and watermelon showing external symptoms were cut into small pieces about 1-2 mm, fixed in 2 % glutraldhyde in 0.1 M Na-Cacodylate buffer,

pH 7.2 and subjected to a vacuum for 1-4 min every 15 min. for 2 hr on ice. Prior to vacuum treatment, floating samples were poked under the buffer surface with pointed metal pokers. Rinsing took place in 0.1 M Na-Cacodylate buffer, pH 7.2, for 45 min, with buffer changes at 15 and 30 min. Further fixation in 1 % Osmium Tetraoxide in Na-Cacodylate buffer, under intermittent vacuum and poking, took place for 1.5 hr. Samples were then rinsed again in the Na-Cacodylate buffer. Dehydrated Samples were dehydrated through an Ethanol series in buffer: 35, 50, 70, 80, 95, 100 and 100 % for 60 min each. Ultra-thin sections were cut using ultramicrotome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Sections were stained with double stain (Uranyl acetate 2 % 10 min followed by Lead citrate for 5 min and examined by a JOEL JM 100-C transmission electron microscope (TEM) (Electron Microscope Unit, EL Azhar University, Cairo, Egypt (Mcdonald and Hiebert, 1975).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from the infected squash leaves was extracted using RNeasy Plant Mini Kit (Qiagen, USA) as in manufacture's protocol. Three µL of RNA were submitted to reverse transcription in a final volume of 20 µL, using 2 µL PCR buffer 10x (0.5 M Tris-HCl pH 8, 0.7 M KCl, 0.1 M $MgCl_2$), 1 µL DTT (100 mmol/µL), 1 µL dNTPs (10 mmol/ µL), 0.5 µL RNase inhibitors enzymes (10 mmol/µL) and 2 µL Reverse-ZYMV primer (100 pmol /µL) ZYMV-Rev, 5′-TGTCTTGGTGGAGGGCTTGT-3′): nucleotide position 290-309 within the nuclear inclusion protein b (NIb) coding region of the virus genome (Accession no.: AB127936), for one hour at 42°C with 0.5 µL MMLV reverse transcriptase (200 mmol/µL). 5 µL of the RT reactions were used for PCR using a 5 µL PCR buffer 10x, 2 µL $MgCl_2$, 1 µL dNTPs (10mmol/µL), 0.5 µL Taq polymerase (5 unit/µL), 1 µL primers (100 pmol) (ZYMV-F, 5′-CCGCCCTCGGAAAAGCT-3′ and ZYMV-R, 5′-TGTCTTGGTGGAGGGCTTGT-3′): nucleotide position 196-309, (primers designed by Gil-Salas *et al*., 2007) depending on genome structure Fig. (1). PCR reactions were performed by a first denaturation of the samples at 94°C for 3 min followed by 35 cycles at 94°C for 30 sec, 43 °C for 30 sec and 72°C for 30 sec and a final elongation step at 72°C for 7 min. PCR products were checked by electrophoresis on 1 % agarose gel (Desbiez *et al*., 2002).

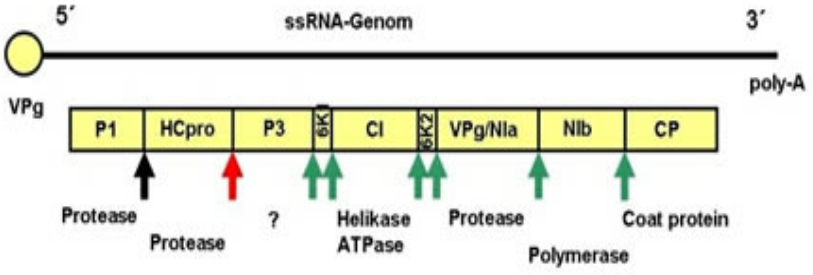

Fig. (1): ZYMV genome map.

Effect of ZYMV on the vegetative growth (fresh weight) and the yield:

Five plants from both the healthy and ZYMV infected plants were chosen to determine the effect of viral infection on the squash plants. Number and weight of fruits and fresh weight of the chosen squash plants were recorded from the beginning of fruiting to the end of experiment (23 days).

RESULTS

Virus isolation and propagation

ZYMV was isolated from the naturally infected squash plants mechanically by single local lesion formed on *C. amaranticolor* after that the isolated virus was inoculated on the systemic host (squash) as a propagative host, which gave severe mosaic, leaf narrowing and leaf deformation Fig. (3).

Host Range

The virus isolated from the naturally infected squash was mechanically inoculated on plants belonging to *Cucurbitaceae*, *Chenopodiaceae*, *Solanaceae* and *Fabaceae* (Table 1).

As shown in table (1) no symptoms were appeared on the plants belonging to family Solanacae. The virus caused systemic infection on the plants belonging to family Cucurbitaceae which it caused severe mosaic and severe leaf deformation on the inoculated *C. pepo* cv. Eskandrani (fig. 3), mosaic on the inoculated *C. sativus* cv. Beta Alpha, severe mosaic and severe leaf deformation on the inoculated *L. acutangula* L. and also on inoculated *C. lanatus* Fig. (4). The virus caused also necrotic local lesions on both *C. amaranticolor, C. quinoa* Fig. (4). Two genera belonging to family *Fabaceae* and the virus caused mild mosaic on *P. vulgaris* and no symptoms were observed on *V. faba.*

Table (1): The reaction different plants species inoculated with the isolated virus**.**

Family	**Test plant**	**External symptoms**
Cucurbitaceae	*C. pepo* cv. Eskandarani	SM, SMl
	Cucumis sativus cv. Beta Alpha	M
	Luffa acutangula L.	SM, SMl
	Citrullus lanatus	SM, SMl
Chenopodiaceae	*Chenopodium amaranticolor*	Chll
	C. quinoa	Chll
Solanaceae	*Datura metel*	--
	D. stramonium	--
	Nicotiana tabacum	--
	N. glutinosa	--
	N. rustica	--
	Gomphrena globosa	--
Fabaceae	*Vicia faba*	--
	Phaseolus vulgaris	M

M= Mosaic, SM= Severe Mosaic, SMl= Severe Malformation (filiform shape) and chll= Chlorotic local lesions

Fig. (2): External systemic viral and viral-like symptoms on the squash plants (*C. pepo* cv. Eskandrani) cultivated in naturally open fields.

Fig. (3): External viral symptoms on *C. pepo* cv. Eskandrani plants mechanically inoculated with the isolated virus, which expected to be ZYMV in the greenhouse.

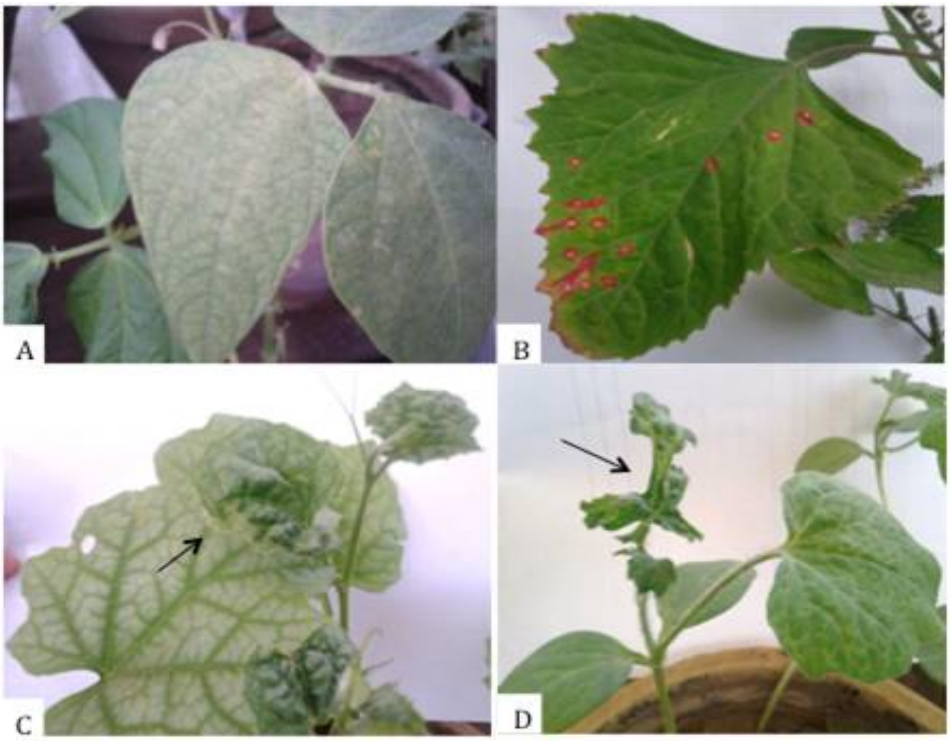

Fig. (4): External symptoms appearing on some inoculated plants as an host range: (A) *P. vulgaris*, (B) *C. amaranticolor*, (C) *L. acutangula* L., and (D) *C. lanatus.*

Physical properties:

Stability of the isolated virus *in vitro* was determined and it was found that the thermal inactivation point (TIP) was 55 C^{o}, dilution end point (DEP) was 10^{-4} and longevity *in vitro* (LIV) was 4 days.

U.V. spectrum of purified Virus

Differential centrifugation followed by sucrose density gradient was used to purify the isolated virus which it was expected ZYMV, table (2) shows the characteristic spectrophotometric data of the purified virus. The maximum and the minimum absorbance of the virus were at 250 and 260 nm respectively. The ratio of A_{280}/A_{260}, A_{max}/A_{min} and 260/280 was 1.0047, 1.099 and 0.99 respectively.

Electron microscopy

Virus morphology

Filamentous flexuous particles, typical of potyviruses, were consistently observed in negatively stained leaf dips preparation. Virus particles in electron microscopy were about 750 nm and 12-15 nm in diameter as shown Fig (5).

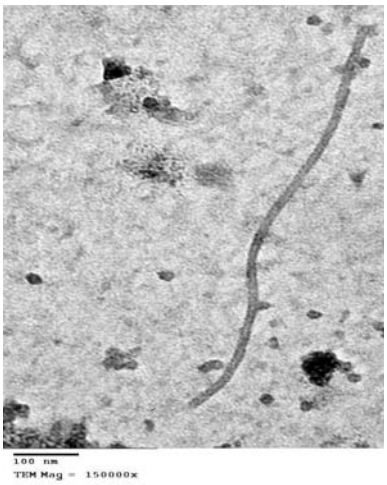

Fig. (5): Morphology of the isolated virus particles as shown under the electron microscope (150000 X).

Table (2): U.V. spectroscopy of purified ZYMV.

A_{260}	A_{280}	$A_{260/280}$	$A_{280/260}$	Max (nm)	Min (nm)	$A_{Max/Min}$	$A_{Min/Max}$	Yield mg/ml
0.212	0.213	0.995	1.0047	250	260	1.099	0.9098	0.883

Ultra thin section

Electron microscopic examination of ZYMV-diseased squash and watermelon mesophyll revealed that the intercellular spaces were very small. The cell wall of infected cells may be thinner than the healthy cells compared with healthy ones. There is degeneration and reduction in the chloroplasts. The chloroplasts are deformed and swollen with reduced lamellar system. Infected squash cells have small intercellular spaces, wavy and destructed cell walls and very small size nucleus, disintegrated chloroplasts with few numbers of starch grains (Fig. 6, A). Nucleus mitochondria appeared in a normal size and shape with no rupture in infected watermelon. The chloroplasts have starch grains like healthy one. The size, shape and arrangement of chloroplasts is normal and similar to the healthy chloroplasts (Fig. 6,B).

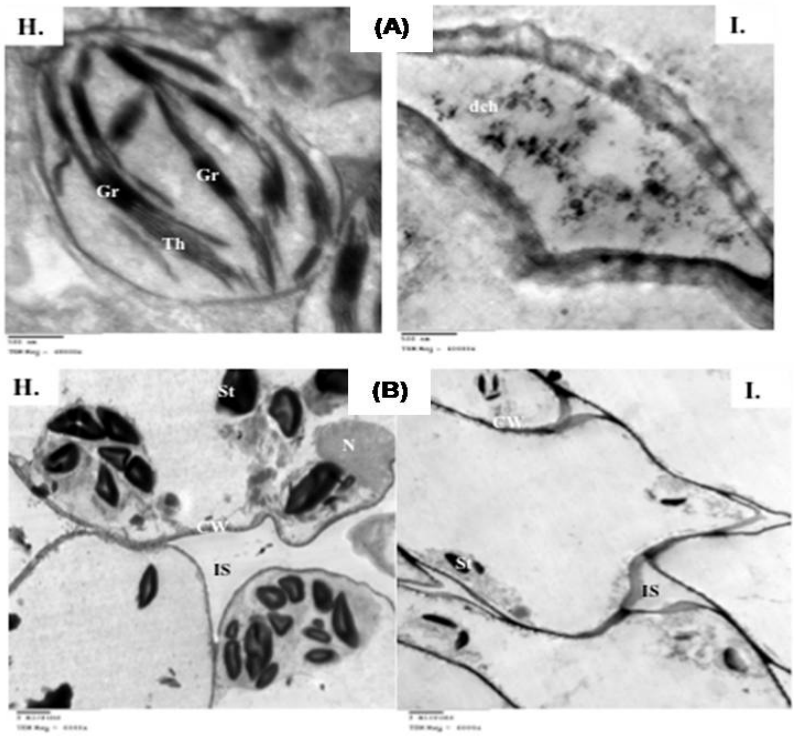

Fig. (6): The electron micrographs of ultra-thin sections of ZYMV infected and healthy squash (A) and water melon leaves (B). Gr= Grana dch= Degraded chloroplast Th= Thillcoides N= Nucleus St=Starch CW= Cell wall IS= Intracellular space

RT-PCR

RT-PCR was used as a powerful technique with widespread application for detection of the virus isolated from the naturally infected squash plants. Specific nucleic acid fragment of the target virus nucleotide position 196-212 within the nuclear-inclusion protein b (NIP) coding region of the virus genome was obtained from fresh or/and dried partially degraded infected squash plants, and it was amplified in the high specific primers (about 20 nucleotides in length). The particular stretch of nucleic acid after amplification (the target sequence) was analyzed by the agarose gel electrophoresis, which resulted in a fragment of 113 bp. (Fig. 7).

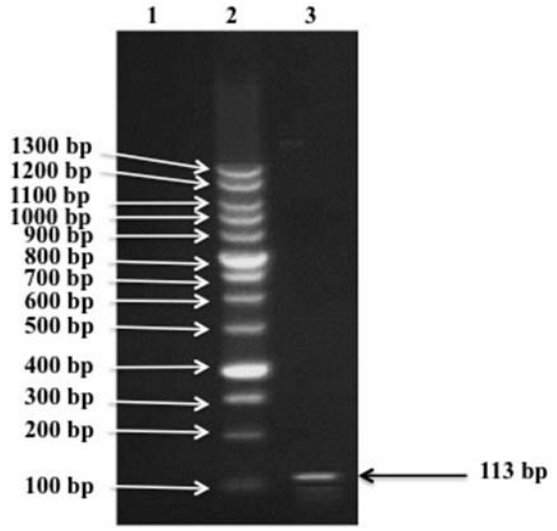

Fig. (7): Agarose gel electrophoresis results of ZYMV RT-PCR products. Lane (1) negative control, lane (2) molecular ladder (100bp) and lane (3) ZYMV–infected sample

Effect of late ZYMV infection on growth weight and yield of squash plants:

Five plants from both the healthy and ZYMV infected plants were chosen at the beginning of the external symptoms appearing for the assaying the effect of ZYMV infection on squash plants cultivated in the open field and detected from ZYMV infection for 18 days from germination. Data showed that, the mean of healthy stem long was 61.2 Cm; whereas it was 39.2 Cm on the naturally infected plant. The mean of vegetative weight of healthy squash plant was 960 g, whereas it was 660 g for the infected. The number of fruits obtained from the healthy were 8 with the total weight of 940 g, but the number of fruits in case infection were 9 fruits with total weight of 770 g.

DISCUSSION

Zucchini yellow mosaic virus (ZYMV), a member of the genus *Potyvirus* in the family *Potyviridae*, was found causing devastating epidemics in commercial cucurbits worldwide (Lisa and Lecoq, 1984). Symptoms of ZYMV include mosaic, yellowing, shoestring, and distortion on leaves; stunting in plant growth; and deformation of fruits.

ZYMV causes one of the most economically important diseases of cucurbit crops. When crops are fully infected before fruit set, severe yield losses of up to 95% occur. These losses reflect reduction in the weight of fruit produced and also quality defects consisting of misshapen and discoloured fruit rendering them unmarketable. Infected fruit also have reduced shelf-life. ZYMV was firstly characterized in northern part of Italy by Lisa *et al.*, (1981).

Our results showed that all of the inoculated plants gave symptoms that are typical for ZYMV infection except *Solanacae* family did not give any symptoms. The common symptoms of the ZYMV infection are local lesions, chlorotic, mosaic, leaf deformation, yellowing and stunting. Necrosis develops in the late stage of the infection and this case was occurred in virus inoculated *C. amaranticolor, C. quinoa* (Lisa *et al.*, 1981). On the same time the all tested *Solanaceae* species were

immune to ZYMV isolate as described by Wang *et al.,* (2004).

The *in vitro properties* of the isolated virus showed that TIP, DEP and LIV were 55 C^o, 10^{-4} and 4 days, and the U.V. spectrum of highly purified particles has the ratio of A_{280}/A_{260} as 1.0047, A_{max}/A_{min} 1.099 and ratio of 260/280 was 0.99. On the other hand electron microscopy of virus particles showed filamentous flexuous particles, 750 nm and 12-15 nm in diameter. Similar results were observed by Abdel-Ghaffar *et al.,* (1998) and Fegla *et al.,* (2009).

The electron microscopy of phytopathic effects revealed that, the yellowing in the late stage of the infection was caused by chlorophyll degradation and the chloroplasts are deformed and swollen with reduced lamellar system as the result of the complex plant pathogen interactions studied by Zechmann *et al.,* (2003) who showed that the number of chloroplasts of ZYMV-infected Styrian pumpkin leaves reduced and its internal structure deformed in comparison with control plants.

Reverse transcription (RT-PCR) for ZYMV was used successfully to amplify viral fragments of the 3' terminal part of the genome, from extracted total plant RNA a fragment of 113 nts was amplified with a specific primer within the nuclear inclusion protein b (NIb) coding region of the virus genome and these results in accordance to (Thomson *et al.,* 1995).

Based on the symptomatology, mechanically transmission, host range, physical properties, electron microscopy and RT-PCR of the virus under study has been identified as a member of Potyvirus group related to ZYMV as described by Brunt *et al.,* (1996) and Van Regenmortel (2000).

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