# Natural Infection of Luffa (*Luffa aegyptiaca* .Mill) with Zucchini Yellow Mosaic Virus in Egypt

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## ABSTRACT

An isolate of zucchini yellow mosaic virus (ZYMV) was obtained from naturally infected luffa plants, showing mosaic, yellowing and blistering symptoms, grown in EL Montaza location, Alexandria governorate. The identification was based on the symptoms developed on diagnostic hosts and serological reactions with antisera to CMV, CGMMV, SqMV, PRSV, WMV-2, ZYMV and TPSV. Luffa isolate of ZYMV was transmitted by *Aphis gossypii, aphis neri* and *Dactynotus sonchi* in non-persistent manner. The virus was purified by centrifugation and PEG. The purified virus had an ultraviolet absorption spectrum typical of a nucleoprotein with A260/280 and A280/260 being 1.07 and 0.93 respectively. Yield of purified virus was 1.15mg/100g infected leaf tissues. Specific antiserum was prepared and found to have a titer of 1:128,000 as determined by indirect ELISA. Electron microscopy revealed pinwheel, scrolls inclusion bodies in the cytoplasm of infected Eskandrani squash cells.

## INTRODUCTION

Zucchini yellow mosaic virus (ZYMV) is a member of family Potyviridae and is considered the most economically important virus attacking cucurbit plants under field conditions. The virus was isolated for the first time in northern Italy (Lisa at el.,1981) and latter from different countries (Lesemann *et al.*, 1983 ;Davis,1986; Al-Musa, 1989; Antignus *et al.*, 1989 and Al-Shawan,1990).

In Egypt, ZYMV was isolated from naturally infected squash plants (Khalil *et al.*,1985; Ibrahim, 1986 ; Awad *et al.*, 1994, Abdel-Ghaffar *et al.*, 1998 and Farag,1999).

luffa plants exhibiting mosaic, yellowing, blister and stunt symptoms were observed in Montaza location, Alexandria governorate. The present study was directed to isolate and identify the causal agent on the bases of symptomology, reactions of diagnostic hosts, serological reaction, insect transmission, photometrical characters of the purified virus as well as electron microscopy of ultrathin sections. Production of specific antiserum to the isolated virus was also carried out.

## MATERIALS AND METHODS

Leaf samples from luffa plants exhibiting mosaic, yellowing, blister and stunting symptoms were collected from Montaza location, Alexandria governorate. Inoculum was prepared by grinding infected leaf tissue in a mortar and pestle, with small amount of 0.02 M phosphate buffer, pH7.0, containing 0.1% 2-mercaptoethanol, leaves of plants to be inoculated were first dusted with carborundum (600 mesh) and then inoculated with a freshly prepared inoculum

using forefinger. The isolated virus was maintained in squash plants that served as a source of the virus for subsequent studies.

**Diagnostic hosts:**

The following diagnostic hosts; *Chenopodium amaranticolor, cucumber (Cucumis sativum* L.) cv.Bita Alpha, lagenaria *(Legenaria siceraria* L.), melon *(Cucumis melo* L.) cv. Ananas, squash *(Cucurbita pepo* L.)cv.Eskandrani, watermelon *(Citrullus lanatus* Thunb.) cv.Giza 1, luffa *(Luffa aegyptiaca* Mill), *Datura stramonium, Gomphrena globosa, Nicotiana glutinosa and Phaseolus vulgaris* cv. Bountiful known to give characteristic symptoms for some viruses affecting cucurbits as recorded by Abdel Ghaffar *et al.* (1998); Awad *et al.* (1994); Huang *et al.* (1993); Ibrahim (1986); Lesemann *et al.* (1983); Lisa *et al.* (1981) and Provvidenti *et al.* (1984) were used for tentative identification of the isolated virus.

**Serological reaction:**

**a) Source of antisera:**

Antisera to cucumber mosaic virus(CMV), cucumber green mottle mosaic virus (CGMMV), squash mosaic virus (SqMV), papaya ring spot virus (PRSV), watermelon mosaic virus 2 (WMV-2), zucchini yellow mosaic virus (ZYMV) and tobacco ring spot virus (TPSV) were used. The antiserum to CMV was locally prepared in our Lab. (Younes, 1995), and ZYMV antiserum was supplied by Agricultural Genetic Engineering Research Institute (AGERI), ARC, Egypt, while CGMMV, SqMV, PRSV and WMV-2 antisera were provided by Agdia and TRSV was kindly supplied by antiserum-Bank, Institute of Seed Pathology for developing countries, Denmark.

**b) Indirect ELISA:**

Indirect ELISA was carried out as described by Fegla *et al.*, (1997), extracts from infected and healthy luffa plants were diluted with coating buffer (0.05 M carbonate, pH 9.6) to 1:10. Wells were coated with antigens by adding 100 µl of to the bottom of the well and incubated for 3 hours at 37°C or overnight at 4°C, The plates were rinsed three times by flooding wells with PBST for 3 minutes each.

Antisera requiring cross-adsorption were diluted 1:500 with filtered extract from healthy tissues diluted 1:20 in serum buffer ( PBS-Tween 20 containing 2% soluble polyvinylpyrrolidone, 0.2% BSA), and incubated for 45 min. at 37°C. The precipitate, which had formed, was removed by centrifugation for 10 min. at 5000 rpm. 100 µl aliquots from the diluted antisera were added to each well, after which the plates were incubated at 37°C for 2 hours or at 4°C overnight, then washed as before.

Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase was diluted 1:1500 in serum buffer, and 100 µl were added to each well, followed by one hour incubation at 37°C, then washed as before.

100 µl of the enzyme substrate, 0.5 mg/ml paranitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8 were added to each well and incubated at room temperature (25°C) for about 30 minutes. The enzyme activity was stopped by adding 50 µl of 3 M NaOH. The ELISA values measured by Multiskan-MS ELISA reader were expressed as absorbency at 405 nm and absorbency values of at least double that of the healthy control were considered positive.

**Aphid transmission:**

For aphid transmission non viruliferous apterous forms of three species of aphids; *Aphis gossypii* Glover, *A. neri* and *Dactynotus sonchi* were starved for one hour in Petri dishes before they were allowed access probes of 3-5 min on infected squash attached leaves. Aphids were transferred in groups of five to each of 10 healthy squash plants and allowed an inoculation period of 10 min. before they were killed by spraying by an insecticide. Inoculated plants were kept under green house conditions and symptoms were observed for one month.

**Purification:**

ZYMV was purified from systemically infected squash plants using the method described by Azzam and Makkouk (1986) with slight modifications. One hundred gm of infected leaf tissues harvested two weeks after inoculation were mixed with 200 ml of an extraction buffer [0.5 M $K_2HPO_4$, 0.002 M $Na_2SO_3$, 0.01 M Na-diethyldithiocarbamate (DIECA) and 0.01 M ethylenediamine-tetraacetate sodium salt (EDTA)] pH 8.5, and homogenized with a warring blender. The homogenate was filtered through 3 layers of cheesecloth, then 3% Triton X-100 was added (v/v). To the homogenate, a mixture of 25% chloroform and 25% carbon tetrachloride (v/v) was added slowly and stirred for 30 min. followed by low speed centrifugation (4000 rpm/20 min). To the aqueous phase, 8% polyethyleneglycol (PEG) , (mol. Wt. 6000) and 3% Nacl (w/v) were added and stirred for one hour at 4°C and subjected to 10.000 rpm/15 min centrifugation using Beckman J-21 centrifuge and Type JA-14 rotor. The pellets were suspended in 0.05 M sodium citrate buffer, pH 7.5 and left overnight at 4°C with occasional stirring. The resuspended pellet was centrifuged at 12.000 rpm for 10 min. Further purification was obtained by a second precipitation with PEG, to the supernatant 8% PEG and 3% NaCl (w/v) were added and stirred for one hour at 4°C and subjected to 12.000 rpm/10 min centrifugation. The obtained pellets were resuspended in sodium citrate buffer and centrifuged at 12.000 rpm/10 min.

Virus presence was checked biologically by inoculating leaves of *C. amaranticolor*. U.V. absorption spectrum of the purified virus at a range of wave length 230-300 nm with 10 nm interval was recorded spectrophotometrically using Shimadzu UV-160 A spectrophotometer. A260/280 and A280/260 as well as virus concentration were estimated. Virus concentration was calculated by assuming an extinction coefficient $E_{260nm}$ of 2.4 (Davis,1986).

### Antiserum production for ZYMV:

Antiserum to ZYMV was produced in New Zealand white rabbit by four weekly intramuscular injection with purified virus. For the first injection, virus at a concentration of 0.25mg/ml was emulsified with an equal volume of Freund's complete adjuvant, and for subsequent injections with Freund's incomplete adjuvant. The rabbit was bled 2 weeks after the fourth injection. The ZYMV antiserum obtained was stored in the presence of 0.05% sodium azide at -20°C.

The titer of ZYMV antiserum was determined by using indirect ELISA as described previously. Extracts from infected and healthy squash plants were diluted with coating buffer to 1:10. Serial dilutions of double fold up to 1:256000 of antiserum from cross- adsorption with filtered extracts from healthy tissues diluted 1:20 in serum buffer were used.

### Electron microscopy:

Tissue samples consisted of small fragments of petioles or mid-vein with adjacent mesophyll tissues excised from healthy and virus infected squash leaves were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.0, for 2 hr at room temperature under vacum . After being rinsed several times with the same buffer, the tissues were post fixed in 1 % osmium tetroxide ($OsO_4$) for 2 hr, then prestained in bulk overnight in 0.5% aqueous uranyl acetate at 4°C. The tissues were dehydrated in an ethanol series, embedded in spurr's epoxy-resin and sectioned 1,0 um thick with a glass knife in a LKB ultramicrotome.

Thin sections for electron microscopy were double-stained in 2% aqueous uranyl acetate for 10 min and lead citrate for 2 min, before examination in a Jeol JEM-100cx electron microscope.

## RESULTS

### Reactions of diagnostic hosts:

Diagnostic hosts reacted with symptoms similar to those produced by ZYMV (Table 1). The virus induced chlorotic local lesions without systemic spread on *C. amaranticolor*, (Fig.1a) and mosaic, yellowing, blistering and deformation on *Cucumis melo* cv. Ananas, *Citrulus lanatus* cv. Giza 1 and *Legenaria siceraria* (Fig.1b,1c and 1d).

Symptoms on *Luffa aegyptiaca* appeared as mosaic yellowing, blistering accompanied with slight to moderate distortion (Fig.2). These symptoms are similar to those observed on naturally infected luffa plants. Squash plants showed severe yellow mosaic and malformation with blistering and shoe sting battern (Fig.3). No symptoms were observed on *G. globosa* , *Phaseolus vulgaris* cv. Bountiful, *Datura stramonium* and *Nicotiana glutinosa*.

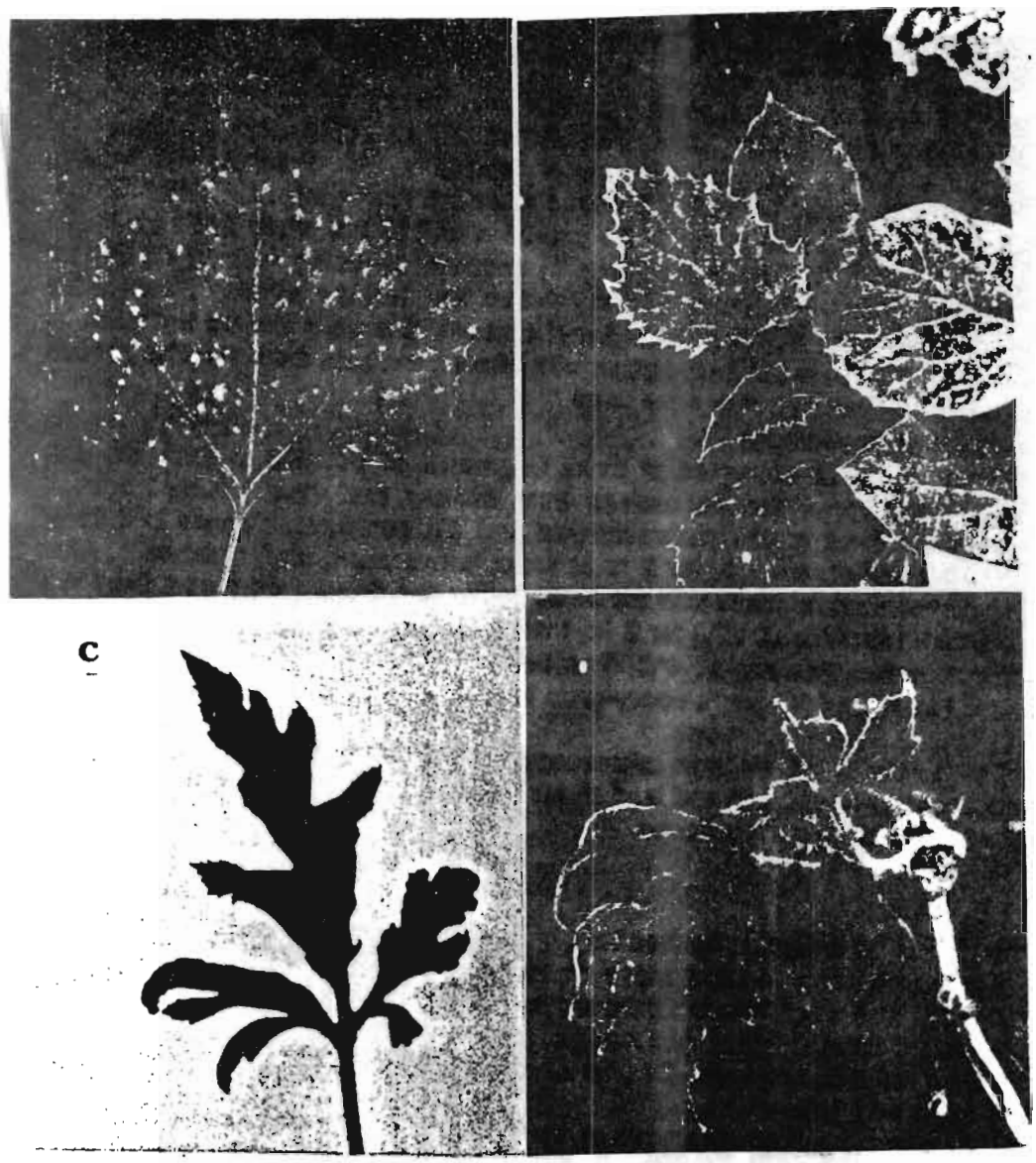


Fig. (1): Symptoms of ZYMV: chlocrotic local lesions on *C. amaranticolor* (a), mosaic, yellowing, blistering and deformation on *Cucumis melo* cv. Ananas (b), *Citrulus lanatus* cv. Giza 1 (c) and *Legenaria siceraria* (d).

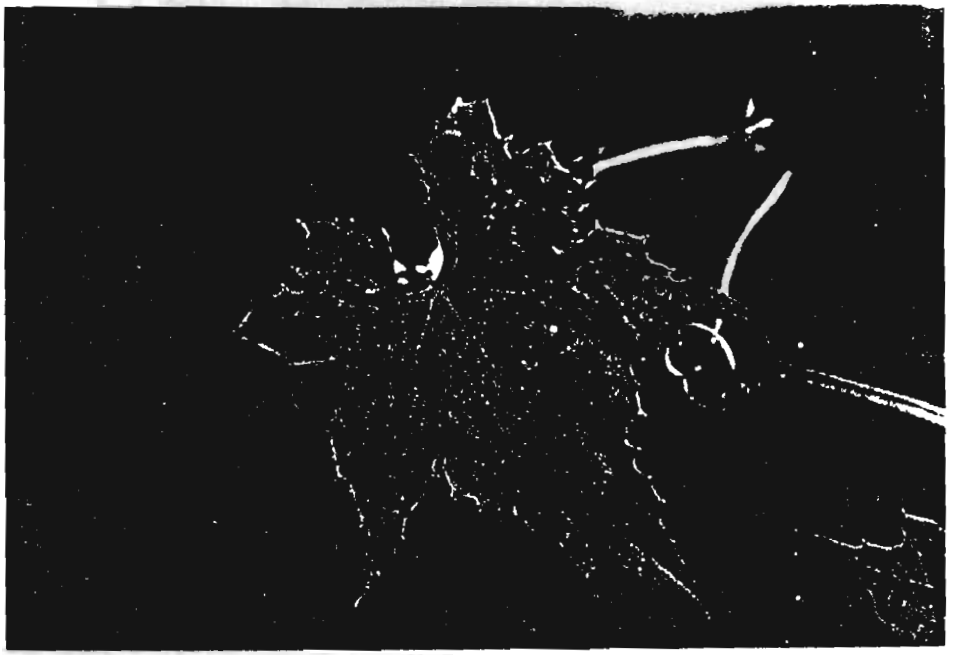

Fig. (2): Yellow mosaic on *Luffa egyptiaca* caused by ZYMV.

Fig. (3): Yellow mosaic and shoe string induced by ZYMV on *Cucurbita pepo* cv.Eskandrani after 3 weeks of inoculation.

Table 1. Reactions of some diagnostic hosts to ZYMV

| Test species | Reactions |
|---|---|
| Fam. Amaranthaceae | |
| *Gomphrena globosa* | – |
| Fam. Chenopodiaceae | |
| *Chenopodium amarenticolor* | CLL |
| Fam. Cucurbitaceae | |
| *Citrulus lanatus* cv. Giza 1 | M,Y,S,D |
| *Cucumis melo* cv. Ananas (melon) | M,Y,S,D |
| *Cucumis sativus* cv.Beta Alfa(cucumber) | M,Y,S |
| *Cucurbita pepo* cv.Eskandrani (squash) | M,Y,S,D,Sh |
| *Legenaria siceraria* (lagenaria) | M,Y,S,D |
| *Luffa egyptiaca* (Luffa) | M,Y,S |
| Fam. Fabaceae | |
| *Phaseolus vulgaris* cv.Bountiful | – |
| Fam. Solanaceae | |
| *Datura stramonium* | – |
| *Nicotiana glutinosa* | – |

CLL= chlorotic local lesion M= mosaic Y= yellowing S= stunting
D=deformation Sh= shoe string

## Serological reaction:

ZYMV antiserum positively reacted with the isolated virus when used at a dilution of 1:500 in indirect ELISA. No positive reaction was detected with antisera to CMV, CGMMV, SqMV, PRSV, WMV-2 and TRSV as determined by indirect ELISA (Table 2).

Table 2. Reaction of infected luffa leaf extract against antisera to CMV, CGMMV, SqMV, PRSV, WMV-2, ZYMV and TRSV as determined by indirect ELISA.

| Luffa leaf extract | Absorbance value at 405 | | | | | | |
|---|---|---|---|---|---|---|---|
| | CMV | CGMMV | SqMV | PRSV | WMV-2 | ZYMV | TRSV |
| I | 0.169 | 0.023 | 0.244 | 0.083 | 0.204 | 0.862 | 0.012 |
| H | 0.095 | 0.019 | 0.174 | 0.087 | 0.129 | 0.275 | 0.042 |

H= healthy I= infected

## Aphid transmission:

The virus was transmitted non persistently by *Aphis gossypii* Glover, *A. neri* and *Dactynotus sonchi* with average transmission rates of 66.7%, 35.7% and 27.2% respectivily when five viruliferous aphids were used on each test plant (Table 3).

Table 3. Transmission of ZYMV by aphid species

| Aphis species | Transmission | |
|---|---|---|
| | Rate* | % |
| *Aphis gossypii* | 8/12 | 66.7 |
| *A. neri* | 5/14 | 35.7 |
| *Dactynotus sonchi* | 3/11 | 27.2 |

* No. of infected plants/No. of tested plants. 5 aphids per plant were used

## Photometrical characters and yield of the purified virus:

The luffa isolate of ZYMV was purified by centrifugation and PEG. Inoculum prepared from such purified virus induced numerous local lesions on inoculated leaves of *C. amaranticolor*. The U.V. absorption spectrum of the purified virus preparation revealed typical spectrum of nucleoprotein. The ratios of A260/280 and A280/260 (uncorrected for light scattering) were 1.07 and 0.93 respectively. The yield of the purified virus was about 1.15 mg/100g fresh weight of squash leaves.

## Production of ZYMV antiserum:

An antiserum against ZYMV was produced. Antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:128000 and not with 1:256000 (Table 4).

Table 4. Indirect ELISA absorbance values (E 405 nm) of extract of ZYMV-infected squash plants in various dilutions of ZYMV antiserum*

| Antiserum dilution | Healthy | infected |
|---|---|---|
| $1:5X10^2$ | 0.275 | 862 |
| $1:10^3$ | 0.141 | 0.554 |
| $1:2X10^3$ | 0.088 | 0.388 |
| $1:4X10^3$ | 0.073 | 0.246 |
| $1:8X10^3$ | 0.054 | 0.148 |
| $1:1.6X10^4$ | 0.036 | 0.088 |
| $1:3.2X10^4$ | 0.026 | 0.058 |
| $1:6.4X10^4$ | 0.019 | 0.042 |
| $1:1.28X10^5$ | 0.010 | 0.022 |
| $1:2.56X10^5$ | 0.009 | 0.015 |

*The experiment was repeated twice and ELISA absorbance values at 405 nm are average of two replicates each

*absorbency values of at least double that of the healthy control were considered positive.

## Electron microscopy:

Electron microscopic examination of ultrathin sections of Eskandarani squash leaves infected with ZYMV revealed the presence of prominent cytoplasmic inclusions. Induced inclusions were composed of bundles, cylinders, tubes and pinwheel figurers. The pinwheels were found to be formed from thin curved arms oriented around a central core with frequently thicker walled scroll-like circles (Fig.4). Electron micrographs indicates that circular inclusions were formed by an extension of the pinwheel arms. The cylindrical inclusions appeared, in longitudinal sections, as bundles of thin plates associated with thick-walled tubes. No inclusions were observed in healthy tissues.

## DISCUSSION

On the basis of symptoms developed on diagnostic hosts and serological reactions with antisera to CMV, CGMMV, SqMV, PRSV, WMV-2, ZYMV and TRSV, using indirect ELISA, the virus isolated from naturally infected luffa plants, grown in Montaza location, Alexandria governorate , with the symptoms of mosaic, yellowing, blisters and stunt symptoms, was identified as ZYMV.

In Egypt, ZYMV was isolated from naturally infected squash plants (Khalil *et al.*,1985; Ibrahim, 1986 ; Awad *et al.*, 1994, Abdel-Ghaffar *et al.*, 1998 and Farag,1999). and this is the first report of natural occurrence of ZYMV on luffa.

ZYMV showed severe mosaic, yellowing, blistering and leaf deformation on most of cucurbits tested. ZYMV was readily sap transmissible to a limited range of hosts, these results are in harmony with the finding of Lisa and Lecoq (1984) and Purciful *et al.* (1994). *Gomphrena globosa* reacted negativily with ZYMV isolated from luffa , the same was found by Al-Shahwan (1990)

ZYMV was transmitted non-persistently by *Aphis gossypii* (Lisa *et al.*, 1981; Awad *et al.*, 1994; Mahgoub *et al.*, 1997 and Abdel-Ghaffar *et al.*, 1998). The present study showed also that, in addition to *Aphis gossypii,* the luffa isolate of ZYMV is transmited by *Aphis neri* and *Dactynotus sonchi* .

The purification of the isolated virus was carried out to determine the photometrical characters of the purified virus and to prepare specific antiserum. The yield of the virus as well as its specific photometrical data such as A260/280 and A 280/260 fall in the range reported for ZYMV (Lisa *et al.*, 1981; Greber *et al.*, 1988; Awad *et al.*, 1994 and Abdel-Ghaffar *et al.*, 1998 ) , however, the slight differences in absorbance ratios as well as yield of the virus of ours and theirs may be attributed to the method used in purification as precipitation by PEG and omission of final step for further purification such as ultracentrifugation or density gradient centrifugation ( Davis, 1986; Hwang and Hseu, 1989; Alhudaib, 1997). Pinweels, scrolls inclusion bodies observed in the cytoplasm of infected squash cells indicated that the virus belongs to the family

Potyviridae . These results were confermed by Lesemann *et al.* (1983) and Abdel-Ghaffar *et al.* (1998).


Fig. (4): Area of cytoplasm from section of squash leaf infected with ZYMV showing pinwheel inclusions (pw), circular inclusion (c) (scroll-like). L, represents a longitudinal section through cylindrical inclusions. T, represents a cross section through cylindrical inclusions

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## الملخص العربي

# تعريف فيروس موزيك الزوكينى الأصفر من لوف مصاب طبيعيا في مصر

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تم عزل فيروس موزيك الزوكينى الأصفر ZYMV من نباتات لوف مصابة طبيعيا وعليها أعراض موزيك أصفر وتشوه بمنطقة المنتزه بالإسكندرية . تم تعريف الفيروس بناء على الأعراض التى أظهرتها العوائل المفرقة وكذلك التفاعل المصلى مع الأمصال المضادة للفيروسات CMV, CGMMV, SqMV, PRSV, WMV-2, ZYMV and TPSV وذلك باستخدام اختبارات الاليزا غير المباشرة. ينتقل الفيروس عن طريق مّن القطن *Aphis gossypii* ومّن الدفله *A. neri* ومن ابرة العجوزه *Dactynotus sonchi* بطريقة غير باقية. تم تنقية الفيروس بالطرد المركزى و البولىايثيلين جليكول PEG وقدر كمية الفيروس النقى فكانت ١,١٥ مليجرام/١٠٠ جرام أوراق نباتات مصابة، وكان أ٢٦٠/أ٢٨٠ وأ٢٨٠/أ٢٦٠ هما ١,٠٧ و ٠,٩٣ على التوالى. أنتج مصل مضاد للفيروس وقدر تركيزه وذلك بإختبار الاليزا الغير مباشره ب ١٢٨,٠٠٠:١ . أظهرت دراسات الميكروسكوب الالكترونى أنواع من الأجسام المحتواه فى صورة طواحين الهواء pin wheels ولفائف scrolls فى سيتوبلازم خلايا نباتات الكوسة المصابة.