Detection of *Cucumber mosaic cucumovirus* in infected cowpea plants (*Vigna unguiculata* L.) from northern Egypt

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Abstract

Cucumber mosaic cucumovirus (CMV) was isolated from naturally infected Cowpea plants (*Vigna unguiculata* L.) showing different symptoms of mosaic; mottle, dwarfing, and vein clearing, grown at certain locations of Alexandria governorate, Egypt, during the growing seasons from 2011- 2012. CMV isolate was transmitted by *Aphid nerii*, *A. gossypii*, and *Myzus persicae* in non-persistent manner. The virus was partially purified using polyethylene glycol (PEG) 6000, and differential centrifugation. The ratio of A 260/280 was 1.622 and A 280/260 was 0.617. Whereas, the ratio of A max/ min was 1.915. Concentration of the virus in the preparation was estimated using an extinction coefficient of E 2600.1% = 5. Yield of purified virus was about 6.88 mg/ 100g fresh weight of leaves of *Nicotiana glauca*. Antiserum titer was determined by Indirect enzyme linked immunosorbent assay (ELISA). Positive ELISA values were obtained up to dilutions of 1: 25600. The virus was detected by indirect ELISA in infected sap at 8, 16 and 24 days after inoculation; and by Tissue blot immunoassay (TBIA) on nitrocellulose membrane after the same period. The unused face of the processed nitrocellulose membrane already printed with plant tissues was tested. Results revealed that both faces of nitrocellulose membrane and Canson paper could be used as solid carriers in TBIA, for detection of CMV in infected leaves. According to Reverse transcription polymerase chain reaction (RT-PCR); the size of amplification of the obtained product was approximately 870 bp for CMV isolate; and was assigned accession number of LN606587. The Phylogenetic tree was generated using partial sequence of CMV isolate, with those of other CMV isolates obtained from GenBank. The aims of the current work were; to produce specific polyclonal antiserum against the purified CMV isolated from cowpea plants, and to register this isolate in GenBank.

Keywords: *Cucumber mosaic cucumovirus*, *Vigna unguculata*, Antiserum titer, Indirect ELISA, TBIA, RT-PCR

1. Introduction

Cucumber mosaic cucumovirus (CMV) infects more than 775 host species in 67 families, and can be transmitted by approximately 75 aphids in a non-persistent manner (Kaper and Waterworth, 1981). It infects cowpea causing systemic symptoms such as mild mottle and mosaic (Thottappilly and Rossel, 1987). In Egypt; CMV was isolated from cowpea (Morsy, 1979; Abd El Aziz, 2015), and was sap-transmitted by aphids in a non-persistent manner (Abd El Aziz, 2015).

CMV played a major role in the deterioration of qualities of many ornamentals as it caused direct damage to the hosts; moreover, it predisposed the plants to secondary invaders (Mahmoud, 2011). Takami *et al*., (2006) reported that infected plants were considered as the main sources of movement and spread of this virus. The first step towards crop protection against CMV infections was through accurate diagnosis of this viral disease, and mapping of its geographical and temporal distribution within an area or crop (Haggag and Kazutaka 2009). According to Stobbs *et al*., (1992); ELISA was the method of choice for detecting and assaying of several plant viruses, because it was sufficiently sensitive for most applications.

Several methods have been described to detect and identify plant viruses such as PCR. Many CMV isolates have been characterized using RT-PCR, Triple antibody sandwich ELISA and Immunocapture (IC) RT-PCR methods (Yu *et al*., 2005). Chen *et al*., (2011) reported that a multiplex RT-PCR assay can detect and differentiate CMV subgroups I and II including their satellite RNA quickly and simultaneously.

The aims of the current work were; to isolate and detect CMV infecting cowpea plants, purification, biological and serological characterization of this CMV isolate. Finally, registration of this CMV isolate in Genbank.

2. Materials and methods

2.1. Isolation of *Cucumber mosaic cucumovirus* (CMV) from cowpea plants

Leaf samples of Cowpea plants showing different mosaic, vain clearing symptoms were separately collected in plastic bags from certain locations of Alexandria governorate during the growing seasons from 2011-2012. Virus inoculum was prepared by grinding infected leaf tissues 1:10 (w/v) with a mortar and pestle in 0.1M phosphate buffer (pH 7.0), containing 0.5% of 2-mercaptoethanol. Leaves of healthy *N. glutinosa* plants in seedling stage were first dusted with carborundum (600 mesh); and then inoculated with a freshly prepared viral inoculum using forefinger method, finally kept in an insect proof greenhouse under observation.

2.2. Diagnostic hosts and symptomology

Several diagnostic hosts including; *Vigna unguiculata*, *Chenopodium amaranticolor*, *Nicotiana glutinosa*, *Gomphrena globosa*, *Vinca rosa*, and *Nicotiana occidentalis* were used to give characteristic symptoms for tentative identification of the isolated virus. Five seedlings of each tested plant species were mechanically inoculated with CMV isolate, and then kept under greenhouse conditions. Plants were examined daily for four weeks for symptoms expression. Inoculated plants that did not show any disease symptoms were cheeked for latent infection by back-inoculation to the indicator host *Chenopodium amaranticolor* (Kaper and Waterworth, 1981; Thottappilly and Rossel, 1987; Hampton *et al*., 1997; Abd El Aziz, 2015).

2.3. Serological diagnosis of CMV isolate

Serological diagnosis was carried out using indirect ELISA.

2.3.1. Source of antisera

Antisera of CMV used in this study were kindly provided by Antiserum Bank, Institute of seed pathology for Developing Countries, Denmark.

2.3.2. Indirect ELISA

Indirect ELISA was carried out as described by Fegla *et al*., (2000). Tested cowpea plant samples were ground in coating buffer (0.05 M carbonate, pH 9.6); and then 100 µl of each sample was added to the bottom of the well, incubated for 2 h at 37^0C or overnight at 4^0C. Plates were rinsed three times by flooding the wells with Phosphate buffer saline + Tween-20 (PBST) for 3 min. each, and then shaked to dry. Wells were blocked by 0.2 % Bovine serum albumin (BSA) in PBS and then incubated for 1 h at 37^0C. The antisera were diluted with healthy plant extract to 1:10, and then recommended dilution (1:500 for CMV antiserum) was made with serum buffer (PBS + Tween-20; containing 2% soluble poly-vinylpyrrolidone (PVP), 0.2% BSA and 0.04% NaN_3). After washing the plates as before, 100 µl of antiserum was added to each well. Plates were incubated at 37^0C for 2 h or at 4^0C overnight, and then washed as before. Goat anti-rabbit gamma globulin IgG conjugated to alkaline phosphatase was diluted 1:1000 in serum buffer. 100 µl were added to each well; followed by one-hour incubation at 37^0C, and then washed as before. 100 µl of the enzyme substrate; 0.7 mg/ ml p-nitrophenyl phosphate in 10 % di-ethanolamine buffer (pH 9.8) were added to each well, and then incubated at room temperature (25^0C) for about 30 min. Enzyme activity was stopped by adding 50 µl of 3M NaOH. ELISA values were expressed as absorbance at 405 nm measured by Multi Skan Ex ELISA Reader. Tested samples were considered positive if their absorbance exceeded twice that of healthy control samples. In each set of the test, wells lacking antigen (coating buffer only) were included as blanks.

2.4. Modes of transmission

CMV was studied for its transmissibility by different methods including;

2.4.1. Mechanical transmission

Cowpea c.v. Kareem7 plants were infected; *N. glutinosa* was used as a virus source, whereas, *Chenopodium amaranticolor* was an assay host. The infected cowpea leaves showing typical disease symptoms were ground 1:10 (w/v) using mortar and pestle in 0.1 M phosphate buffer (pH 7.0), containing 0.5% 2-mercaptoethanol. Leaves of healthy *N. glutinosa* plants were first lightly dusted with carborandum (600 mesh), and then rubbed with forefinger previously soaked in the freshly prepared inoculum according to Hamza *et al*., (2018).

2.4.2. Aphid transmission

Three species of aphids were tested for their ability to transmit isolates of CMV. These aphid species were; *Aphis gossypii* Glover, *A. nerii* Boyer and *Myzus persicae* Sulz. (These aphids were identified by Entomology Department, Faculty of Agriculture, Alexandria University, Egypt). A pterous forms of aphids were starved for one hour then allowed to feed on CMV-infected cowpea leaves for 3-5 min. They were then transferred to 10 healthy *N. glutinosa* seedlings at the rate of 10 aphids/ plant, and then left for 5 min. as an inoculation feeding period. Aphids were then finally killed with an aphidicide; Malathion (0.1%). Plants were kept under insect proof cages, and observed carefully for symptoms development. Percentage of successful transmission was recorded in each case (Fegla *et al*., 2000).

2.5. Purification of CMV isolate

CMV was purified according to the method of Walkey, (1991). One hundred grams of fresh systemically infected *N. gluaca* leaves were collected 16-20 days after inoculation. They were homogenized with 200 ml of 0.5 M sodium citrate buffer (pH 6.5); containing 0.2 ml thioglycollic acid, and 200 ml of chloroform. Operations should be carried out in a cold room at $4^°$C and in a refrigerated centrifuge. The mixture was homogenized for several minutes until a fine

homogenate was produced. The homogenate was centrifuged at low speed (5000 g) in a bench centrifuge for 15 min. The aqueous supernatant was pipetted off and retained; then the pellet and chloroform were discarded. 10 % (w/v) polyethylene glycol (mol. wt. 6000) was added to the supernatant and shaked until dissolved, and then left to stand for 30 min. The supernatant was centrifuged at 5000 g for 20 min. then discarded; whereas, the pellet was retained. The pellet was re-suspended in 0.05 M citrate buffer (pH 7.0) containing 2% Triton X-100; and then 3.5 ml of buffer was added to each 35 ml capacity centrifuge tube, left overnight. Mixture was centrifuged at 15000 g for 20 min. The supernatant was retained while the pellet was discarded. The supernatant was centrifuged at 75000 g for 150 min. in an ultracentrifuge. Supernatant was discarded while the pellet was retained. The pellet was re-suspended in 1 ml of 0.05 M citrate buffer at pH 7.0, left for several hours. The re-suspended pellet was centrifuged at 5000 g for 10 min.; the supernatant was retained, whereas the pellet was discarded. The supernatant contained the partially purified virus which might be subjected to further sucrose gradient separation if required. Virus presence was checked biologically by inoculation of *Chenopodium amaranticolor*. UV. absorption spectrum of the purified virus at a range of wavelength 230-320 nm was recorded spectrophotometrically; using ultra violet spectrophotometer Jon Way 6405 UV/VIS A 260 / 280, A 280 / 260 and A max / A min. Virus concentration was calculated by assuming an extinction coefficient E 2600.1% of 5 at 260 nm (Noordam, 1973; Eni *et al.*, 2010). An outline of this procedure used for CMV purification is illustrated in Fig. (1), according to Abd El Aziz, (2015).

2.6. Production of CMV polyclonal antiserum

An antiserum against CMV was prepared according to Chalam *et al.*, (1986). A male white New Zealand rabbit over two Kg was injected intramuscularly four times at 10 days intervals with one ml containing 3 mg of purified CMV, and emulsified with an equal volume of Freund's incomplete adjuvant. Ten days after the last injection; the blood was collected from the marginal ear vein and allowed to clot, and then the antiserum was separated and stored frozen until required. The separated antiserum was clarified by centrifugation at 5000 g/ 35 min.; 0.2% NaN_3 was added, and then antiserum was divided in aliquots, kept frozen until needed for different serological assays. Antiserum titer was determined using indirect ELISA as described by Fegla *et al.*, (2000). Extracts from infected and healthy *N. glutinosa* plants were diluted with coating buffer to 1: 10. Serial dilutions of double fold up to 1:256000 of antiserum from cross absorption, and filtered extracts from healthy tissues diluted 1: 20 in serum buffer, were used according to Younes, (2003).

2.7. Serological detections

2.7.1. Detection of CMV in infected plant leaves after different periods of inoculation, by tissue blot immunoassay (TBIA) and indirect ELISA

CMV infected *N. glutinosa* leaves after different periods of mechanical inoculation; 1, 2, 3, 4, 5, 6, 8, 16 and 24 days, were detected by TBIA and indirect ELISA. CMV antiserum was used at dilution of 1:500 in serological tests. Samples of apparently healthy and infected *N. glutinosa* plants were checked serologically for virus presence by TBIA as described by Lin *et al.*, (1990), and modified by Fegla *et al.*, (2000). Tissues of rolled leaves; stems and roots from healthy and infected plants were cut with razor blades. Exposed cut edges were pressed on nitrocellulose membrane (NCM 0.45 nm, BIO-Rod Laboratories, Richmond, CA), previously dipped in 0.05M carbonate buffer (pH 9.6), and then placed on filter paper for 5 min. to dry. Treated membranes were then placed in a glass Petri plates containing 10 ml of blocking buffer (2% BSA in phosphate buffer saline (PBST), pH 7.0), gently agitated for 1 h. The membrane was removed from the blocking solution; dipped in dist. water, and then transferred to a virus antiserum (CMV was diluted to 1:500 in PBST).
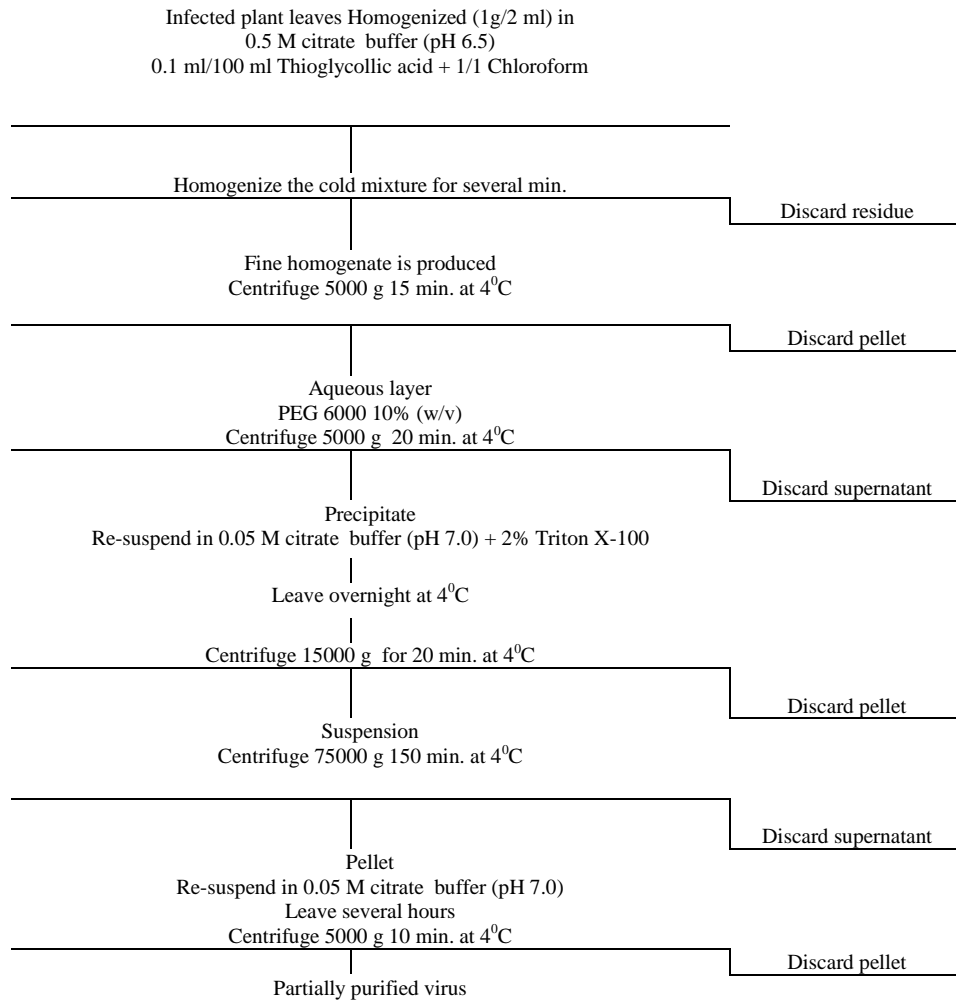
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Infected plant leaves Homogenized (1g/2 ml) in
0.5 M citrate buffer (pH 6.5)
0.1 ml/100 ml Thioglycollic acid + 1/1 Chloroform

Homogenize the cold mixture for several min.

Discard residue

Fine homogenate is produced
Centrifuge 5000 g 15 min. at 4^0C

Discard pellet

Aqueous layer
PEG 6000 10% (w/v)
Centrifuge 5000 g 20 min. at 4^0C

Discard supernatant

Precipitate
Re-suspend in 0.05 M citrate buffer (pH 7.0) + 2% Triton X-100

Leave overnight at 4^0C

Centrifuge 15000 g for 20 min. at 4^0C

Discard pellet

Suspension
Centrifuge 75000 g 150 min. at 4^0C

Discard supernatant

Pellet
Re-suspend in 0.05 M citrate buffer (pH 7.0)
Leave several hours
Centrifuge 5000 g 10 min. at 4^0C

Discard pellet

Partially purified virus

Fig. 1: Diagrammatic outline of CMV purification by infected *N. gluaca* leaves

The membrane was gently agitated for 2 h; removed from the first antibody solution, dipped in dist. water, and then washed twice by agitation for 10 min. in phosphate buffer + Tween-20 (PBST). The membrane was dipped in dist. water; transferred to a 1:1000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase in PBST, and then gently agitated for 1 h. Finally; the membrane was removed from the second antibody dilution; dipped in dist. water, and then washed twice in TBST for 10 min. each. A substrate solution of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium (NBT); was made during the final washing, in which membranes were incubated for color development. After color development, the reaction was stopped by washing the nitrocellulose membrane in 0.01M Tris-HCl containing 0.05M EDTA (pH 7.5). Positive reaction of TBIA was indicated by the development of purple color on the blots. On the other hand, negative reaction developed no color or green color. Virus presence was detected after different periods of mechanical inoculation (1, 2, 3, 4, 5, 6, 8, 16, and 24 days) by indirect ELISA as previously described.

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2.7.2. Possibilities of reducing the cost of TBIA by using alternative solid carrier instead of nitrocellulose membrane

Comparison between regular types of solid carriers such as Canson paper (150 g/m^2), and nitrocellulose membrane was carried out using TBIA. Tissues of rolled leaves; stems and roots from healthy and infected *N. glutinosa* plants were cut with razor blades, then exposed cut edges were pressed onto regular types of Canson paper and nitrocellulose membrane. Both faces of the nitrocellulose membrane and Canson paper were printed by the plants as described by Al-Khalaf *et al.*, (2009). The rest of the procedure was followed as previously described with TBIA.

2.8. Molecular detection of CMV by RT-PCR

Two specific primers of CMV were synthesized by Bio-Basic Inc., Canada. The sequences of the reverse and forward primers used for CMV detection are shown as described by Rizos *et al.*, (1992). The upstream primer 5'CP (5' CTCGAATTCGGATCCGCTTCTCCGCGAG 3') corresponded to nucleotides 1149 to 1161 of CMV-Q RNA 3. Whereas; the downstream primer 3'CP (5' GGCGAATTCGAGCTCGCCGTAAGCTGGATG GAC 3') corresponded to bases 1998 to 2015 of CMV-Q RNA 3 (Davies and Symons, 1988), which was common to the 3' ends of the other CMV-RNA species. Synthesis of cDNA of the virus coat protein (CP) gene was based on the method given in the Thermo Scientific Verso 1-Step RT-PCR kit using the following parameters; 50°C for 15 min. (1 cycle) to allow reverse transcription to be performed, verso inactivation at 95°C for 2 min. (1 cycle), followed by 35 cycles at 95°C for 1 min., 55°C for 45 sec., 72°C for 1 min., followed by 7 min. of incubation at 72°C using a Hybaid thermal reactor (Abd El Aziz, 2015). Sequences of the CMV-CP genes that were translated to the amino acid sequences were later submitted to the NCBI nucleotide sequence database, GenBank. Comparison between CMV proteins and other corresponding strains proteins

was generated as described by Thompson *et al.*, (1994).

3. Results

3.1. Disease symptoms observed on different plants leaves induced by CMV

Serological detection of infected plants revealed the involvement of a virus namely *cucumber mosaic cucumovirus* (CMV) isolated from cowpea plants showing mosaic (Fig. 2a); mosaic blisters and severe distortion on *N. glutinosa* (Fig. 2 b), same symptoms on *N. occidentals* (Fig. 2c), and mosaic on *Vinca rosa* (Fig. 2d). Necrotic local lesions with red border were observed on inoculated leaves of *C. amaranticolor* (Fig. 3a); whereas, *Gomphorena globosa* plant leaves exhibited necrotic local lesions in addition to systemic mosaic (Fig. 3b).

3.2. Modes of transmission

3.2.1. Mechanical transmission

CMV was easily transmitted mechanically using 0.1 M phosphate buffer (pH 7.0) on cowpea and/or *N. glutinosa* plants; respectively, with ratio of 100%.

3.2.2. Aphid transmission

CMV was transmitted non-persistently by three aphids spp. named; *Aphid nerii*, *A. gossypii* and *Myzus persicae* with average transmission rates of 80%, 50% and 90%; respectively, when five aphids were used for each test plant (*N. glutinosa*) (Table 1).

3.3. Ultra violet absorption spectra of purified CMV

The absorption spectrum of the purified virus isolate determined through Jon Way 6405 UV/VIS spectrophotometer, was typical for nucleoprotein (Fig. 4). The ratio of A 260/280 was 1.622 and A 280/260 was 0.617; whereas, the ratio of A max/min was 1.915. Concentration of the virus in the preparation was estimated using an extinction
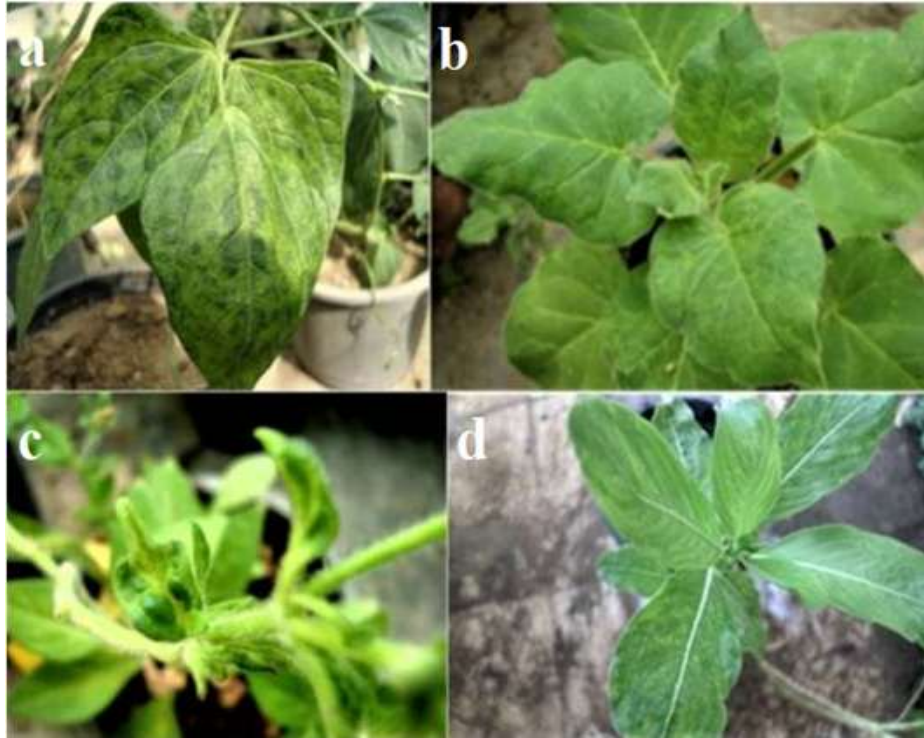
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Fig. 2.: a)-Symptoms caused by CMV on cowpea (*Vigna unguculata*) leaves cv. Kareem7 leaves showing mottling mosaic, b)-Mild mosaic appeared on *N. glutinosa* leaves induced by CMV, c)-Symptoms caused by CMV on *N. occidentallis* leaves showing mosaic, blisters and deformation, and d)-Mosaic and yellowing caused by CMV on *Vinca rosa* leaves.
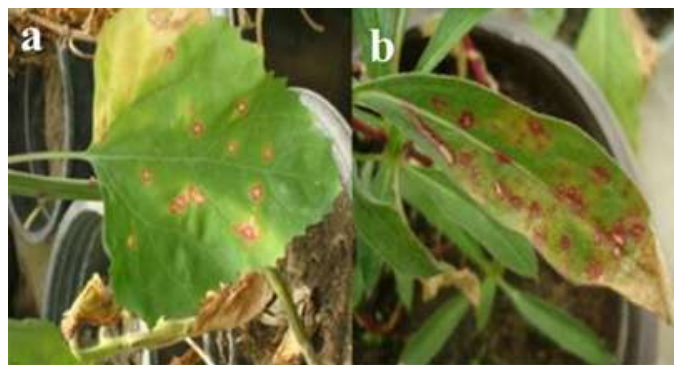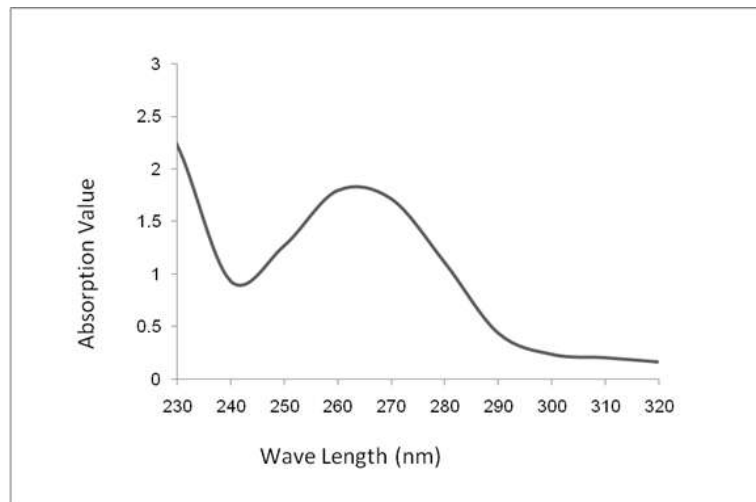

Fig. 3.: a)-Necrotic local lesions with reddish margin observed on *C. amaranticolor* leaves induced by CMV, b)-Necrotic local lesions caused by CMV on inoculated leaves of *Gompforena glbosa*.

Table 1: Transmission of CMV by several Aphid spp.

Aphis species	Transmission	
	Rate*	%
Aphid nerii	8/10	80
A. gossypii	5/10	50
Myzus persicae	9/10	90

Where; *Number of infected plants/ no. of tested plants, 10 aphids were used per plant

Fig. 4: Ultra Violet absorption spectrum of CMV purified from *N. glauca* infected leaves

coefficient of E2600.1% = 5. Yield of the purified virus was about 6.88 mg/100 g fresh weight of *N. glauca* leaves. The ultra violet absorption spectrum of the collected virus had A minimum at 240 nm, and A maximum at 260 nm. When the purified virus was tested biologically on *C. amaranticolor* leaves, numerous local lesions were observed.

3.4. Production of purified CMV antiserum

An antiserum against CMV isolate was produced. The antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:25600, and not with 1:51200 (Table 2).

3.5. Detection of CMV in infected plants after different periods of inoculation

Indirect ELISA and TBIA were used to detect the isolated CMV in infected *N. glutinosa* plants at different periods (1, 2, 4, 8, 16 and 24 days), after mechanical inoculation. Dilutions of 1:10 in carbonate buffer (pH 9.6) from CMV infected *N. glutinosa* plants were also tested. Results showed that CMV antiserum 1:500 could detect the virus in infected sap at 8, 16 and 24 days after inoculation by indirect ELISA (Table 3), and by TBIA on nitrocellulose membrane (Fig. 5a).

Table 2: Indirect ELISA of CMV extract from infected cowpea plants in various dilutions of CMV antiserum*

Antiserum dilution	Infected	Healthy
$1{:}1{\times}10^2$	2.625	0.966
$1{:}2{\times}10^2$	1.784	0.813
$1{:}4{\times}10^2$	1.462	0.682
$1{:}8{\times}10^2$	1.172	0.549
$1{:}1.6{\times}10^3$	1.015	0.503
$1{:}3.2{\times}10^3$	0.752	0.338
$1{:}6.4{\times}10^3$	0.642	0.311
$1{:}1.28{\times}10^4$	0.633	0.302
$1{:}2.56{\times}10^4$	0.500	0.216
$1{:}5.12{\times}10^4$	0.468	0.278

Where; *The experiment was repeated twice, and the indirect absorbance values at 405 nm were average of two replicates each. Absorbance value double that of the healthy control was considered positive

Table 3: Indirect ELISA absorbance values at 405 nm, for CMV in 1:10 dilution of sap extracted from infected plants at different periods after inoculation

Days after inoculation	Indirect ELISA absorbance values (E 405 nm)
24	0.674
16	0.611
8	0.587
4	0.499
2	0.421
1	0.351
H	0.274

3.5.1. Possibility of using processed nitrocellulose membrane

The unused face of processed nitrocellulose membrane already printed with plant tissues on one face, was tested for detection of CMV in infected *N. glutinosa* leaves using TBIA. Results indicated the possibility of using the empty face of nitrocellulose membrane even after 3 months from processing for detection of the CMV, since it gave pronounced purple color with the infected leaf tissues (Fig. 5b).

3.5.2. Possibility of using both faces of the solid carrier

Results demonstrated that both faces of nitrocellulose membrane and Canson paper could be used as solid carriers in TBIA for detection of CMV in infected *N. glutinosa* leaves and stems Fig. 5(a, b). Positive reactions of both tests on both faces of solid carriers were clearly observed.

Fig. 5a: a): Sensitivity of TBIA for detection of CMV in infected *N. glutinosa* plants after different periods of mechanical inoculation on nitrocellulose membrane. b): TBIA used for detection of CMV on the unused face of previously processed nitrocellulose membrane. Where; I: infected, H: healthy
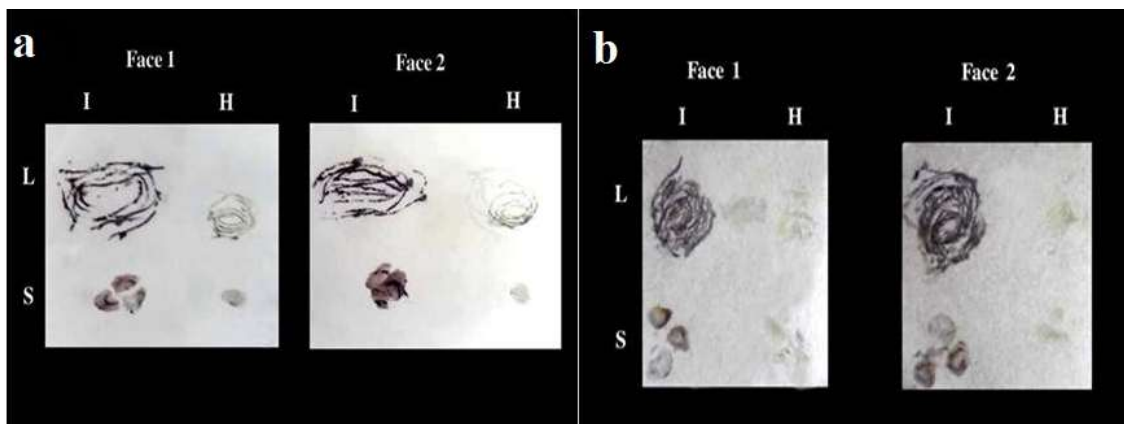

Fig.5b: a): TBIA used for detection of CMV in infected leaves and stems of *N. glutinosa* plants on both faces of nitrocellulose membrane at the same time. b): TBIA used for detection of CMV in infected leaves and stems of *N. glutinosa* plants on both faces of Canson paper at the same time. Where; L: leaves, S: stems, I: infected, H: healthy

3.6. Molecular studies

3.6.1. Detection of CMV by RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed on total RNA extracted from infected cowpea leaf tissues showing infection with the CMV isolate. Results obtained confirmed the serological diagnosis, and the specificity of the primers used in this study. The size of the obtained amplification product was approximately 870 bp for CMV (Fig. 6).

3.6.2. Nucleotide sequence studies of CMV-CP gene

Forward and reverse primers were designed from the coat protein gene sequence of the CMV genome. Results obtained confirmed the specificity

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of the primers used in this study. For CMV; the PCR amplified product was of expected size of approximately 870 bp long. Nucleotide sequencing of CMV-CP gene was carried out on the purified RT-PCR products. This CMV nucleotide sequence was later submitted to NCBI database GenBank, then accepted and received accession number of LN606587. A phylogenetic tree was generated using partial nucleotide sequence of the CMV isolate, and those of other CMV isolates obtained from GenBank (Fig. 7).
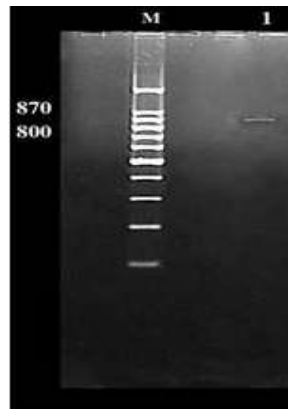

Fig. 6: Agarose gel electrophoresis showing the RT-PCR amplification of CMV isolate coat protein gene. Where; M: DNA Marker (1000 bp), and Lane 1: CMV (870 bp)
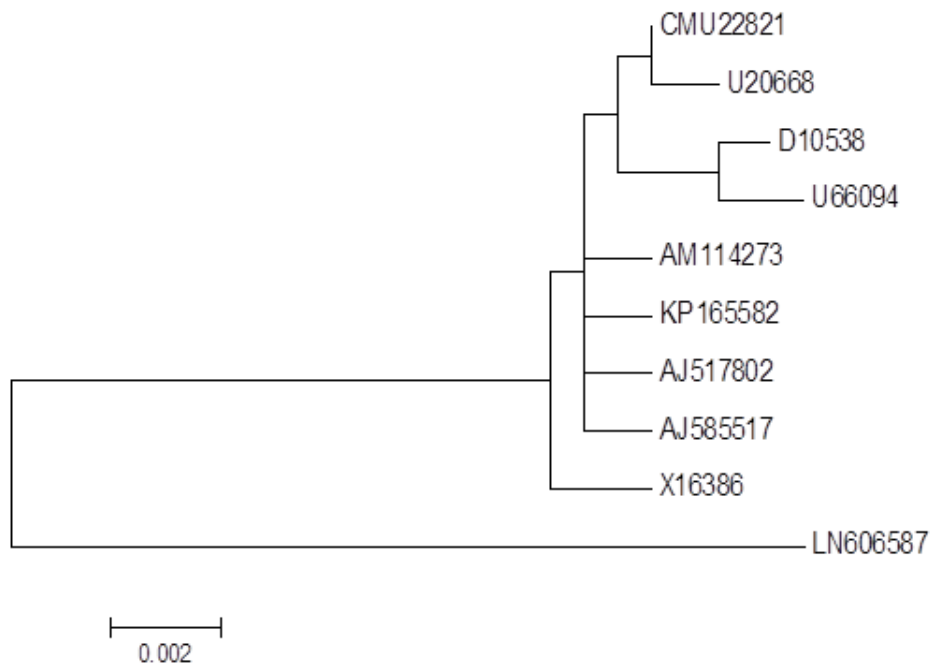

Fig. 7: Phylogenetic tree generated using partial nucleotide sequence of CMV isolate with those of other CMV isolates obtained from GenBank

4. Discussion

CMV caused infection of Cowpea plant under field conditions, this plant was considered however as one of the most economically important Fabaceae crops cultivated in different regions of Egypt (Morsy, 1979). CMV can be transmitted by mechanical inoculation, and was sap-transmissible also by aphids in a non-persistent manner (Dheepa and Paranjothi, 2010). Purification of the isolated CMV was carried out to determine the photometrical characters of this isolate, and to prepare its specific antiserum. Yield of CMV as well as its specific photometrical data such as; A260/280, A280/260 and A mix/min, fall in the range reported previously for CMV by Chalam *et al*., (1986); Eni *et al*., (2010). The titre of the antiserum obtained for CMV was 1: 2.56×10^4; similar results were obtained by Eni *et al*., (2010).

TBIA has been used by many investigators for surveys, diagnosis and detection of viruses in different plants parts. It has several advantages such as; cheaper in cost, could be completed in less than four hours without sacrificing sensitivity, do not require sophisticated facilities, and was sensitive enough to detect the virus in all parts of infected plants as reported by Lin *et al*., (1990); Makkouk and Kumari, (1996); Fegla *et al*., (2000); Fegla *et al*., (2003). Data concerning possibility of using alternative solid carriers instead of nitrocellulose membrane such as Canson paper (150 gm/m^2) in TBIA, revealed noticeably higher signal from CMV diseased material as compared to healthy leaf samples of *N. glutinosa*; moreover, it gave pronounced purple color by infected leaf tissues with TBIA. Canson paper was equally sensitive as the nitrocellulose membranes. Similar conclusions have been reported by Fegla *et al*., (2001b), and by other investigators working with Dot blot immunobinding assay (DBIA) mainly; Sherwood, (1987); Heide and Lange, (1988). Concerning the use of both faces of the nitrocellulose membrane and the Canson paper

as solid carriers by TBIA; they detected the CMV in infected *N. glutinosa* leaves, through giving pronounced purple color with these infected leaf tissues. Such results agreed with those reported by Al-Khalaf *et al*., (2009) who worked on Bean yellow mosaic virus (BYMV). Evaluation was carried out for detection of CMV after different periods of its inoculation into *N. glutinosa* leaves. Results revealed that TBIA was more sensitive than indirect ELISA, since they detected CMV after 2 and 4 days of inoculation, respectively. Such results however did not agree with those reported by Abd El-Aziz, (2000); Fegla *et al*., (2001a), who found that indirect ELISA was more sensitive than DBIA with CMV and *Potato Y Potyviruses* (PVY).

Previous studies of Hadidi *et al*., (1995); Thompson *et al*., (1995) illustrated the importance and usefulness of PCR as a molecular diagnostic tool of plant viruses. CMV isolated from cowpea plants was identified on the basis of serological reaction by indirect ELISA, and by using of specific oligonucleotide primers in RT-PCR. Analysis of PCR products on agarose gel electrophoresis revealed amplification of specific bands of approximately 870 bp, as similarly recorded by Rizos *et al*., (1992); Younes, (2011). After that CMV was assigned an accession numbers of LN606587. These results were in agreement with those reported by Anderson *et al*., (1995); Henderson *et al*., (2014); Megahed *et al*., (2014). RT-PCR was extremely sensitive and detected single infected leaf among 99 healthy leaves; in contrast to ELISA which detected only one infected leaf among nine healthy leaves (Gillaspie *et al*., 2001).This one-step RT-PCR protocol was rapid and sensitive in routine diagnostic laboratories as reported by Kumari, (2009).

Conclusion

Cowpea plants were infected with *Cucumber mosaic cucumovirus* (CMV) under field conditions,

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which caused great losses in this crop. We studied CMV transmission and the host ranges which acted as alternative host plants, to avoid the increase in disease incidence. Preparation of the specific antiserum gave us the chance to detect the infected samples in large scales. Moreover, registration of this CMV isolate in GenBank will provide any future investigator with the map of CMV distribution in Egypt.

Conflict of interests

The authors declare that there are no conflict of interests.

Acknowledgments

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