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Molecular Characterization of *Cucumber mosaic virus* (CMV) local isolates in Libya

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Abstract

Field observation revealed that the most common symptoms on naturally infected plants in Al-Marg City- Libya were severe mosaic, mild mosaic on Cucumber, pepper, tomato and eggplant. These symptoms were suspected of being caused by *Cucumber Mosaic Virus* (CMV) and confirmed by The Reverse transcription-polymerase Chain Reaction (RT-PCR). The test isolates demonstrated RT-PCR amplification product of 540 bp, Pure RT-PCR product of 2 Libyan CMV isolates (3TR_Tomato, 5CF_cucumber) were sent for sequencing. Comparison of coat protein sequences of two Libyan isolates from tomato and cucumber plant to that of other reported isolates in genbank, Both sequences received accession numbers of OM000303 and OM000304 from Genbank, The phylogenetic tree generated from alignment based on the nucleotide sequence comparison of the CMV coat protein of the 2 Libyan isolates CMV isolates and the 13 CMV isolates from different geographical areas showed by MEGA X, revealed that our Libyan isolates (OM000303 and OM000304) are clustered together in the same branch but there was a higher genetic difference between two Libyan isolate in the same clade. Libyan isolates are out grouped to other isolate in the tree, this is mean that we may got new CMV isolates.

Keywords: *Cucumber mosaic virus* (CMV), Cucumber, Tomato, RT-PCR, Phylogenetic analysis.

Introduction

Cucumber mosaic virus (CMV) is the type member of the genus *Cucumovirus*, family Bromoviridae (*1*). CMV was first discovered in 1916

(*2*). It is one of the most devastating plant viruses with a worldwide distribution and a very wide host range of plant species including important vegetable crops (*3, 4*). CMV is found everywhere cucurbits are grown, and it is most active in temperate and subtropical zones (*5*). Worldwide, CMV is one of the five major viruses affecting field-grown vegetables (*6*). Its host range extends to more than 1,200 plant species belonging to over 100 families of dicotyledonous and monocotyledonous angiosperms (*7-9*). It usually causes diseases on plant in Cucurbitaceae, Solanaceae, Brassicaceae, and Fabaceae and various kinds of symptoms are produced by CMV on these plants (*10-12*). CMV infection causes varied symptoms, commonly mild to severe mosaic, vein clearing, vein banding and malformation (*13*). And also it causes leaf distortion, puckering of leaves, stunting, yellow discoloration, shoestring, necrotic rings spot on leaves and fruits (*14-16*), as well fruit lesions, which result in reduced yield by 33 to 60% (*17-19*). CMV is efficiently transmitted by more than 80 species of aphids including *Myzus persicae* and *Aphis gossypii* in a styletborne, nonpersistent manner (*20, 21*). And it is highly infectious and can be easily transmitted to other plants after handling of infected plant materials or during cultivation (*4*). The genome of CMV is a single-stranded, positive-sense consists of tri-segmented, RNAs (RNA1, RNA2 and RNA3) and sub-genomic RNA4 derived from RNA3 encoded coat protein gen. The size of these RNAs 3357,3050,2120 and 876 nucleotides respectively (*22, 23*). The numerous strains of CMV have been classified into two major groups, I and II, according to serology, nucleic acid hybridization and nucleotide sequence similarity (*24-26*). Then, another researcher divided subgroup I into IA and IB based on gene sequences and phylogenetic analysis of CP open reading frame (ORF) of RNA3 (*27*). The subgroups are not evenly distributed across agricultural regions. Subgroups IA and II have a worldwide distribution, while subgroup IB is reported to be principally restricted to Asia.
In Libya, the CMV virus was recorded on tomato plant in Al-Zawia (*28*) and AL-Lusayta AL-Jabal AL-Akhdar (*29*). And furthermore from pepper and squid plants, in different area in the middle region of AL-Jabal Al-Akhdar, and from wild tobacco in AL-Jabal Al-Akhdar and western Costal-belt of Libya (*30, 31*). All since studies used biological characters including symptomology, host range, aphid transmission and polymerase chain reaction for dictation and characterization of CMV isolates. The aim of this study determinant the nucleated sequences of coat protein gen in two CMV isolates from cucumber and tomato plants.

Materials and methods

Virus source

Four samples of plant levees showing mosaic symptoms were collected form cucumber, eggplant, pepper and tomato cultivated in private farm from AL-Marg area at AL-Jabal AL-Akhder-Libya. Each sample was divided into several samples and placed in plastic bags. These samples were sent to Departement of Botany and Microbiology, Faculty of Science, University of ALexandaria-Egypt for farther molecular work.

Extraction of viral RNA from host plant leaf tissue:

Total RNA was extracted from leaf samples using the Thermo scientific Gene JET Viral RNA purification kit (Lot:00135006, GE Healthcare, UK).

Reverse transcription-polymerase Chain Reaction (RT-PCR) for the detection of CMV

First-strand cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (Lot:00160714, Thermo Scientific) and its buffer (5X) [50mM Tris-HCl (pH 8.3 at 25 Co), 250 mMKCl, 20 mM MgCl2, and 50 mM DTT] in presence of reverse primer were used. 1µl of RNA was added to (4 µl (5x) RT-Buffer, 2 µl (10 mM) dNTPs,1 µl of primer, 1 µl (200 u/ µl) of RT-enzyme, 1 µl (20 u/ µl) Ribo Lock RNase Inhibitor, 12 µl H2O). The mixture was incubated at 42 Co for 60 minutes. After cDNA synthesis, the reverse transcriptase was inactivated at 70 Co for 15 min in the PCR thermocycler (Gene Amp 9700 thermocycler, Applied Bio-system ABI, USA). Viral cDNAs were amplified by PCR in a reaction mixture (25 µl final volume) containing 5x Mg-free Buffer, 25 Mm MgCl2 (Promega), 5 nmol/ml of each primer ,10 mM dNTP mixes (Promega), 5U/µl Taq DNA polymerase (Promega) and 5 µl of the RT mixture. **Table 1** lists specific synthetic oligonucleotides as primers used, region amplified, expected product size, and reference for each test. Thermal cycling conditions, such as incubation time, number of cycles, and temperatures during PCR amplification were shown in **Table 2 (*32*).**

Agarose gel electrophoresis of PCR-amplified DNA:

According to Sambrook and Russell (*33*), 1% agarose gel was prepared by

dissolving 0.5g agarose powder in 50ml of 1x TBE buffer (10x TBE Buffer:107.80g Tris base, 55g Boric acid, and 7.44g Disodium EDTA.$2H_2O$ pH 8.3 and the final volume were brought to 1liter with distilled water). The running buffer used was 1x TBE .5 µl of the PCR amplification products were mixed with 3 µl 6x gel loading buffer (38% Sucrose, 0.1% Bromophenol blue, and 67mM EDTA), and run in the 1% agarose gel in 1xTBE buffer. Electrophoresis was performed at 80V, the gel was stained with ethidium bromide solution (0.5µg/ml) for 10 min, visualized on an ultraviolet transilluminator (UVP) (M-20, upland, USA), and photographed using a gel documentation system (Alpha-ChemImager, USA). The size of the PCR product was determined using a 100bp and 1Kb DNA ladder (Promega).

Table 1: Specific synthetic oligonucleotides primers used for cloning CMV isolate genes.

Primer Name	Primer sequence (5'-3')	Region Amplified	Expected product size (bp)
CP F	GCGCGAAACAAGCTTCTTATC	coat protein	540
CP R	GTAGACATCTGTGACGCGA		

Table 2: Thermal cycling conditions used for PCR protocol for CMV

Final extension	Elongation	Annealing	Denaturation	Cycles	Initial Denaturing
72 Co /10 min	72 Co /2min	55 Co /2min	94 Co /1min	(40)	94Co / 4min

Sequencing of 2 CMV Libyan isolates 3TR_Tomato, 5CF_cucumber

Pure RT-PCR products of 2 Libyan CMV isolates (3TR_Tomato, 5CF_ cucumber) were sent for sequencing to the sequencing facility MWG-Biotech AG, Germany. Comparison of coat protein sequences obtained of two Libyan isolates from tomato and cucumber plant, and other reported isolates, were submitted to the GenBank.

Results

Symptomology and virus detection of naturally infected plants:-

The naturally infected plants exhibited symptoms including severe mosaic, mild mosaic, mosaic and intervinal yellowing, chlorotic spots, **Figure 1.** Mosaic and decreased leaf area and mosaic and intervinal yellowing suspected of being *Cucumber mosaic virus* (CMV) symptoms.These symptoms were visually observed on naturally infected cucumber, tomato, pepper and eggplant plants at different locations within in city Al-marg. The causal virus of the naturally observed symptoms suspected of being caused by CMV was identified based on symptomology and reverse transcription polymerase chain reaction (RT-PCR).

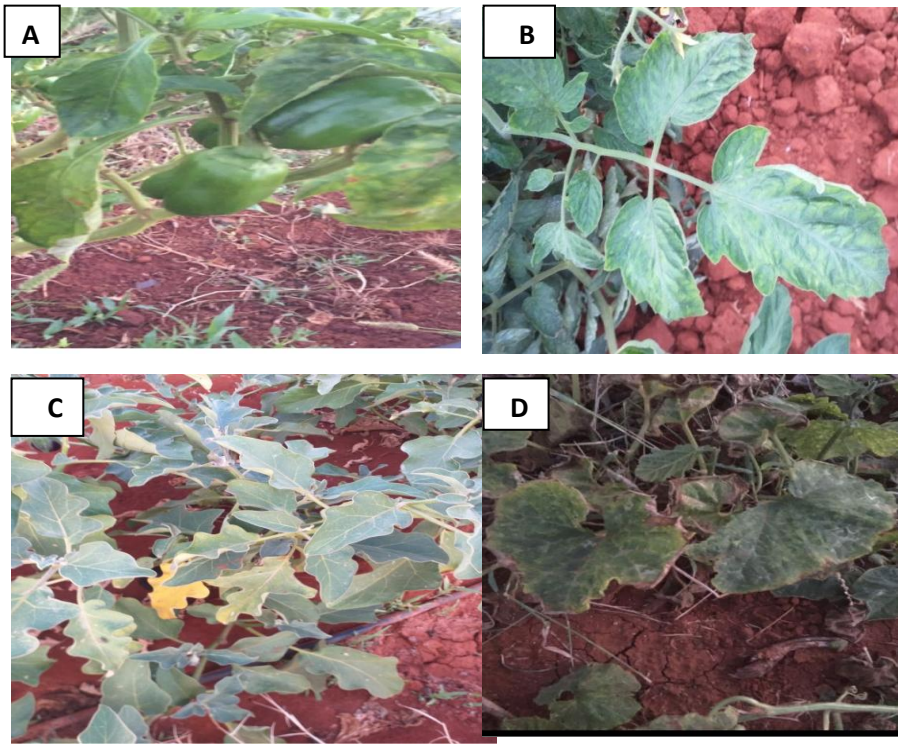

Fig. 1: Virus-related symptoms CMV surveyed fields in Al-Marg City of Libya in 2021. A: mosaic and interveinal yellowing in peper; B: Chlorotic

spots, mosaic and decreased leaf area in Tomato; C: mosaic and intervinal yellowing in eggplant; D: severe mosaic in cucumber.

Polymerase Chain Reaction (RT-PCR) for the detection of CMV in 4 Libyan Samples:-

The result of PCR that produced by the using of coat protein primers for the four isolates were showed in Figure 2. The result revealed the production of bands of 540bp. representing the virus isolates. This result indicated that these virus isolates belongs to CMV.

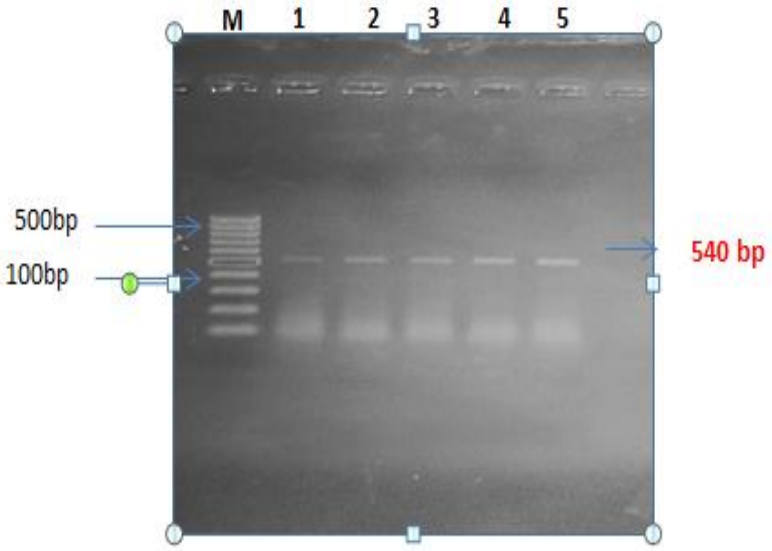

Fig. 2: Electrophoretic mobility of DNA amplicons obtained by Polymerase Chain Reaction (PCR) from total RNA of host leaves samples using primer pair (CMVF RNA3/ CMVR RNA3). Approximately aliquots (8µl) of Libyan isolates were run through 1% agarose gel in 1xTBE buffer stained with ethidium bromide and photographed. Lane m: Molecular weight standard 100bp DNA ladder (Promega); lanes (1-4): different host tissue samples lane 1: cucumber, lane 2: pepper, lane 3: tomato, lane 4: eggplant samples.

Comparison of nucleotide sequence of the two Libyan isolates of CMV coat protein gene to that of other recorded CMV isolates:-

By the use of the sequences obtained for coat protein gene they were compared to similar protein products of other CMV serotype (1a, 1b). The accession number and assigned abbreviations of these isolates are listed in **Table 3**. A pairwise comparison of partial coat protein sequence of CMV Libyan isolate GenBank accession (OM000303 and OM000304), showed 86 % to 92 % identity at the nucleotide level to other isolated on GenBank. Also, Libyan isolates 3TF showed higher sequence identity against isolates 79-13 and Salzlandkreis-2_18 from Serbia and china, respectively. Libyan isolates 5CF showed higher sequence identity against isolates 581-11 and 79-13 from Serbia.

Table 3: Accession numbers, country of origin and serotype of coat protein sequences of CMV isolates used in multiple sequence alignment and phylogenetic analysis

Accession Number	Isolate	Geographical origin	Serotype	Sequence reference
JN054635	CLW2	Malaysia	1b	NCBI
EF593023	Amaranthus	India	1b	Srivastava et al. (2004)
HE962480	Vir	France	1b	NCBI
AJ810259	KS44	Germany	1b	Deyong et al. (2005)
JX025995	Esf172	Iran	1b	NCBI
AJ829778	BAR92/1	Spain	1b	Bonnet et al. (2005)
KC527752	RP24	South Korea	1b	NCBI
KC527746	RP17	South Korea	1b	NCBI
EF216865	YN	China	1b	NCBI
D10538	Fny	Not stated	1a	Owen et al. (1990)
D16405	Iizuka	Japan	1a	Karasawa et al. (1997)
AJ276481	Mf	South Korea	1a	NCBI
HM015286	BB8	Taiwan	1a	Deng et al. (2010)
OM000303	3TR	Libya		This study

OM000304	5CF	Libya		This study

Phylogenetic analysis illustrating evolutionary relationship between the Libyan CMV isolates and other reported CMV isolates

Phylogenetic and molecular evolutionary analyses were conducted from alignment of the partial nucleotide sequences of CMV Libyan isolate to that of other isolates from different geographical areas using MEGA X, (Bootstrap test of phylogeny (1000 replicates), Neighbor-Joining method (34). The phylogenetic tree generated from alignment based on the nucleotide sequence comparison of the CMV coat protein of the 2 Libyan isolate CMV isolates and the 13 CMV isolates from different geographical areas showed by MEGA X, (Bootstrap test of phylogeny, Neighor-Joining) is shown in **Figure 3** revealed that our Libyan isolates (OM000303 and OM000304) are clustered together in the same branch but there was a higher genetic difference between two Libyan isolates in the same clade. Libyan isolates are out grouped to other isolate in the tree, this is mean that we may got new CMV isolates.

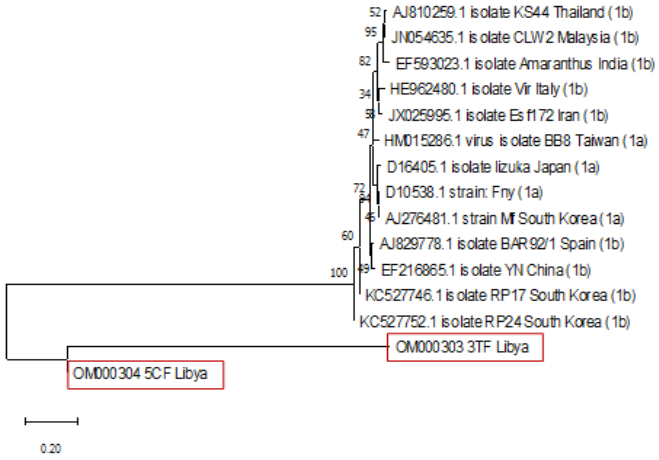

Fig. 3: Nucleotide tree Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to

infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. This analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 858 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [4].

Discussion

Detection of the origin and phylogenetic relationship of CMV Libyan isolate was essential as not much reports of its occurrence in Libya are known and also for understanding the relationship of this isolate to others previously described, genes were selected for PCR amplification sequencing. Sequencing results confirmed that the PCR products obtained with the primer pair (CMV RNA3F/ CMV RNA3R) were indeed the coat protein gene. Nucleotide sequences of Egyptian CMV isolates were chosen and submitted to the NCBI nucleotide sequence database, GenBank. All sequences were deposited in the GenBank with accession numbers (OM000303, OM000304). The result of Reverse transcription-polymerase Chain Reaction (RT-PCR) by using the CP-specific Reverse primer to detect CMV and production bands of 540 bp revealed that the virus isolate was CMV (*34*). Previous studies detected a recombination between subgroups I and II as well as IA and IB in RNA 3 based on phylogenetic and computational analysis (*32, 35*).

Developing the detection of CMV using the Luminex xTAG technology based on nucleic acid test. The advantage of this technology is allowing the simultaneous detection of several targets. The presence of CMV and differentiation between the two subgroups I and II for which significant differences with regard to virulence and severity of symptoms have been proved by this method. The phylogenetic tree generated from alignment based on the nucleotide sequence comparison of the CMV coat protein of the 2 Libyan CMV isolates and the 13 CMV isolates from different geographical areas showed by MEGA X, (Bootstrap test of phylogeny, Neighor-Joining) revealed that our Libyan isolates (OM000303 and OM000304) are clustered together in the same branch but there was a higher genetic difference between two Libyan isolates in the same clade. Libyan isolates are out grouped to other isolate in the tree; this is meaning that results may get new CMV isolates.

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التوصيف الجزيئي لفيروس موزاييك الخيار (CMV) المعزولة من ليبيا

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المستخلص العربي

أظهرت الملاحظة الميدانية أن الأعراض الأكثر شيوعاً على النباتات المصابة طبيعياً في مدينة المرج في ليبيا كانت االموزاييك الشديدة واالموزاييك المعتدلة على الخيار والفلفل والطماطم والباذنجان. تم الاشتباه في أن هذه الأعراض ناجمة عن فيروس موزاييك الخيار (CMV) وتم تأكيدها من خلال تفاعل سلسلة البوروليميريز (RT-PCR). أظهرت عزلات الاختبارحزمة بطول 540 قاعدة مزدوجة ، وتم إرسال منتج RT-PCR النقي لعزلتين من عزلات CMV الليبية (3 TR_Tomato، 5CF_cucumber) للتسلسل. ومقارنة تسلسلات الغطاء البروتينى لعزلتين ليبيتين من نبات الطماطم والخيار مع عزلات أخرى تم الإبلاغ عنها في بنك الجينات ، تلقى كلا التسلسلين أرقام انضمام OM000303 و OM000304 من Genbank ، شجرة النشوء والتطور الناتجة عن المحاذاة بناءً على مقارنة تسلسل النيوكليوتيدات لـ CMV أظهر الغطاء البروتتينى لعزلتين من العزلات الليبية من عزلة CMV و 13 عزلة CMV من مناطق جغرافية مختلفة أظهرتها MEGA X أن عزلاتنا الليبية (OM000303 و OM000304) متجمعة معًا في نفس الفرع ولكن كان هناك فرق وراثي أعلى العزلة الليبية فى نفس الفرع. يتم تجميع العزلات الليبية في عزلة أخرى في الشجرة ، وهذا يعني أننا قد نحصل على عزلات جديدة من الفيروس الموزاييك الخيارCMV.

الكلمات المفتاحية : فيروس موزاييك الخيار و التوصيف الجزيئي.