Annual Research & Review in Biology

35(4): 45-52, 2020; Article no.ARRB.54366
ISSN: 2347-565X, NLM ID: 101632869

Serological and Molecular Characterisation of *Cucumber mosaic virus* Infecting *Lagenaria siceraria* L. in Adim, Biase L.G.A., Cross River State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author ATO designed the study and wrote the protocol. Author OIE handled data collection, performed the statistical analysis and wrote the first draft of the manuscript. Author AAJM managed the literature searches. Author EEE managed the analyses of the study. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2020/v35i430211
Editor(s):
(1) Dr. Oluyomi A. Sowemimo, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
Reviewers:
(1) Moataz Eliw, Al-Azhar University, Egypt.
(2) Ferreira, Luiz Leonardo, Universidade Estadual da Paraiba, Brazil.
Complete Peer review History: http://www.sdiarticle4.com/review-history/54366

Original Research Article

Received 29 November 2019
Accepted 03 February 2020
Published 02 June 2020

ABSTRACT

Cucumber mosaic virus (CMV) is one of the most important viral pathogens infecting a wide range of plant species in Nigeria. Mosaic and mottle symptoms were observed on *Lagenaria siceraria* L. in Adim Southern-Nigeria in 2018 and the infected leaves collected for investigation. This research was aimed at characterising the virus responsible for this infection with a view to identifying it. Antigen coated plate (ACP) enzyme linked-immunosorbent assay (ELISA) and gene sequencing were employed methods in the characterisation process. The Amplified Complementary Deoxyribonucleic Acid (cDNA) was cloned and the nucleotide sequence was determined. Result of serology revealed that the virus belonged to the genus *Cucumovirus* while the gene sequence

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obtained when compared to known virus sequences present in the GenBank using the Basic Local Alignment Search Tool (BLAST) program available at National Centre for Biotechnology Information (NCBI) revealed 97% sequence homologue with *Cucumber mosaic virus* confirming it as *Cucumber mosaic virus*. This is the first report of CMV infecting *L. siceraria* in Nigeria. I recommend further studies on insect and host range test of this virus be carried on.

Keywords: Cucurbitaceae; RT-PCR; Cucumovirus; Amplicon; Potyvirus.

1. INTRODUCTION

The Family Cucurbitaceae comprises of about 95 genera and 965 species*. Lagenaria siceraria* L. commonly called bottle gourd is one of the important vegetable crops in this family, it is one of humankind's first domesticated plants, providing food, medicine and a wide variety of utensils and musical instruments [1].

Lagenaria siceraria has been reported to be threatened by diseases of fungal, bacterial and viral origin [2] and pests, such as the pumpkin caterpillars (*Diaphania* spp.) and the melon fly (*Bactrocera cucurbitae* Coquillet). These have constituted serious constraints to the cultivation of this plant [3-6]. Aphids, whiteflies and thrips can become serious limitations to the production of *L. siceraria* through the extensive damage they provoke and also through the transmission of viral disease [7-9].

Plant viruses are ranked the most common causal agents of diseases infecting *L. siceraria* worldwide [10]. *Cucumber mosaic virus* is the type member of the genus Cucumovirus (family Bromoviridae) reported to infect *L. siceraria*. The size of CMV genome is approximately 8 kb consisting of three linear positive-sense single stranded RNA molecules [11] which are packaged in separate icosahedral virions. The virus is mechanically transmissible and also by aphids in a non-persistent manner [12] and is divided into two subgroups (I and II) based on nucleotide sequence identity and serological properties and is reported to infect over 1,000 species in more than 85 botanical families [13]. Since CMV has an extensive host range, there are numerous records of susceptible species causing severe economic losses. Therefore, mosaic and mottle symptoms observed on this plant are suspected to be caused by CMV (Plate 1).

Several viruses infecting cucurbits have been characterized and identified in different parts of Nigeria. [14] reported infection of cucurbits in Northern Nigeria, [15] reported infection of cucumber by Potato ring spot virus (PRSV) and infection of *Cucurbita moschata* by Moroccan watermelon mosaic virus (MWMV) in Calabar Southern Nigeria, [16,17] characterised *Yam mosaic virus* (YMV) and *Tobacco mosaic virus* (TMV) infecting cucurbits in Northern Nigeria. [18] provided a checklist of viruses prevalent in South West Nigeria. However, studies regarding virus infecting *L. siceraria* are lacking in Nigeria. This study was aimed at characterising the virus responsible for the infection of *L. siceraria* with a view to identifying it.

Plate 1. Mosaic and mottle symptoms on *Lageneria sceseraria*

2. MATERIALS AND METHODS

2.1 Source, Isolation and Maintenance of Virus Isolate

Infected leaf sample showing symptoms of mosaic and mottle was isolated from *L. siceraria* in Adim, Biase Local Government Area of Cross River State, Nigeria in 2018 and maintained through mechanical inoculation on carborundum (600 mesh) dusted leaves of *Cucumeropsis mannii* and were thereafter used as source of inoculum.

2.2 Preparation of Virus Inoculum and Maintenance

Virus inoculums were prepared by triturating symptomatic virus infected leaf tissues of *L.*

siceraria in pre-sterilized cold pestle and mortar in the inoculation buffer and maintained on young seedlings of *Cucumeropsis mannii* in the screen house of the University of Calabar, Calabar, Nigeria.

2.3 Serological Tests

Antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) as described by [19] was used to determine the genus to which the virus isolate belong to. The symptomatic leaf sample of *L. siceraria* of 0.1 g was triturated in 1 ml of coating buffer (0.015 M Na_2 Co_3 + 0.0349 M $NaHCo_3$ + dH_2O) and dispensed into ELISA plates. After incubation at 37°C for 1 hour the plate was washed 3 times with phosphate buffer solution (PBS-Tween) for 3 min between each wash. Cross adsorption was made by grinding 1 g of healthy plant sample in 20 ml of conjugate buffer (^{1}/2 PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP). Antisera to CMV and the universal potyvirus antiserum were diluted at 1:3000 in the adsorption solution and 100 µL of each antiserum polyclonal antisera were added to the wells of the ELISA plates and again incubated at 37°C for 1 hour. The ELISA plates were then washed 3 times with phosphate buffer solution (PBS-Tween). One hundred µL of protein, A-alkaline phosphatase conjugate diluted in the ratio 1:15000 in conjugate buffer (^{1}/2 PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP + 0.02 g $NaNO_3$) was added per well and the plates incubated at 37°C for 1 hour. The plates were again washed 3 times with PBS-T. One hundred-µl of 0.001 g·ml^{-1} of *p*-nitrophenyl phosphate substrate in substrate buffer (97 ml diethanolamine + 800 ml H_2O + 0.2 g $NaNO_3$ and HCl to give pH 9.8) was added per well and incubated at room temperature for 1 hour. For all incubations, plates were covered with ELISA cover plates to avoid edge effects and to maintain uniform temperature. Healthy plant samples were used as controls. Absorbance ($A_{405\ nm}$) was measured after 1 h of incubation, using an ELISA plate reader (Micro Read 1000 ELISA Plate Analyser, U.S.A). The samples were considered positive when the ELISA reading was at least twice the reading for the healthy control [19].

2.4 RNA Extraction from Infected Leaf Samples

Total RNA was isolated from the infected leaf sample of *L. siceraria* using a cetyltrimethylammonium bromide (CTAB) protocol as described by [20]. One hundred milligram of the infected leaf sample was grounded in sterile mortar and pestle using 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% CTAB) (hexadecetyltrimethylammonium bromide); and 0.4% β- mercaptoethanol, added just before used. The sap was poured into a new 1.5 ml Eppendorf tube. The tubes were vortexed briefly, incubated in a 60^{o}C water bath for 10 minutes, and then allowed to cool to room temperature. Then 0.75 ml of phenol chloroform isoamyl (25:24:1) was added to each tube containing the sap. Each tube was vortexed vigorously to form an emulsion and then centrifuge at maximum speed of 12000 rcf for 10 minutes.

The supernatant was then transferred to a clean 1.5 ml tube. Three hundred of cold isopropanol was added to the supernatant to precipitate the nucleic acid (RNA) and mixture kept at -80^{o}C for 10 minutes. The mixture was centrifuge at 12,000 rcf for 10 min to precipitate the nucleic acid. The supernatant was discarded and the nucleic acid pellet washed in 500 µl of 70% ethanol and centrifuge at 12,000 rcf for 5-10 minutes. The ethanol was decanted and the resultant nucleic acid pellet was air-dried at room temperature. Nucleic acid pellet was then re-suspended in 50 µl sterile distilled water and used as a template source for RT-PCR. Nucleic acid extracts from the leaves of healthy plants were used as negative control, whereas nucleic acid extracts from infected leaves samples with known potyvirus infection were used as positive control.

2.5 Reverse Transcript Polymerase Chain Reaction (RT-PCR)

Virus-specific cDNA fragment was amplified from total nucleic acid derived from the infected leave sample by a RT-PCR method as described by [21]. RT-PCR was performed using the CMV primer Forward 5'GGIVVIGTIGGIWSIAARTCIAC3', Reverse 5'ACICCRTTYTCDATDATRTTIGTIGC 3' as described by [22]. The RT-PCR reaction mixture (50 µl) consisted of I µl each of CICP 5' and CICP 3', 5x Go Taq green buffer (10.0 µl), $MgCl_2$ (3.0), dNTPs (1.0 µl), Reverse transcriptase (0.24 µl), Taq DNA polymerase (Promega) (0.24 µl), sterile distilled water (30.52 µl), nucleic acid (1:10 dilution) (3.0 µl).

Amplifications were carried out in a GeneAmp 9700 PCR system thermalcycler (Applied

Biosystem Incorporated, USA) using the following thermocyclic conditions; 42°C for 30 minutes for reverse transcription, 94°C for 3 minutes for initial denaturing, followed by 40 cycles of denaturing at 94°C for 30 s, an annealing step at 40°C for 30 s, an extension at 68°C for 1 minute and a final extension at 72°C for 10 minutes ended the RT-PCR reaction. The PCR reaction products were separated on 1.5% agarose gel, subsequently stained with ethidium bromide, visualized in UV light and photographed RT-PCR assay produced a PCR amplicon of expected size (approximately 500 bp).

2.6 Amplicon Purification and Sequencing

The RT-PCR amplicon was purified, 95% ethanol was added to 40 µl of the amplicon in a new 1500 µl Eppendorf tube and solution kept in -80° C for 10 minutes. The tube was centrifuged at maximum speed for 10 minutes and the supernatant discarded. Five hundred of 70% ethanol was added and centrifuge at maximum speed for 5 minutes, the supernatant was discarded and the tube left in room temperature to dry the purified cDNA after which the purified product was dissolved in 30 µl of sterile distil water. The product was then sent to International Institute for Tropical Agriculture (IITA) bioscience laboratory for sequencing.

2.7 Sequence Analysis

The sequences were compared to known viral sequence using BLAST program available at National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence identities were calculated from the "sequence identity matrix" option in MEGA 6 window software.

3. RESULTS

3.1 Antigen Coated Plate (ACP) Testing against Potyvirus and *Cucumovirus antisera*

The results obtained from this test revealed that the virus isolate reacted positively against the CMV antiserum, there was negative reaction against the universal potyvirus antiserum (Table 1).

3.2 Gene Sequence and Sequence Alignment

Fig. 1 represents nucleotide sequence of the virus isolated from *Lagenaria siceraria.*

Results as shown in Fig. 2 revealed gene alignment of the virus isolate showing 97% sequence homologue with *Cucumber mosaic virus*, Sequence ID: MG021460.1. 97%.

Table 1. Antigen Coated Plate (ACP) Enzyme Linked Immunosorbent Assay (ELISA) for detection of *Cucumber mosaic virus* (CMV) and Potyviruses

Samples	Location	OD reading at $A_{405\ nm}$ against Virus Polyclonal Antibodies	
		CMV	Potyvirus
Lagenaria siceraria virus isolate	Adim	0.894*	0.562
Healthy Control		0.326	0.405
Infected Control		2.687	1.894

**Sample was considered virus positive when the optical density (OD) reading at $A_{405\ nm}$ was 2x greater than the absorbance from healthy controls*

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AGATAAAGGAATGGGGGACTTCGACGGTTTGTAGATTACCGCCAACTCAGGTCCCGCCTAAAGGAGGG

ACTCCGCTAAGCACGAGCAATACATTCGGCGATTAAGTCGCCGCCTAACATACCACAATACTCATAGC

GGAAGGTGACCTTATGTCTAGTCAAAGCGACAAGACAATAAGAAAATTTCTTAAATAGGTCACACTTT

GTGCTCTTTAATCTTACCAAAGTAACGTGGTCCTCAGAAATACCCTGGGACTCATGCACAGTTTTGAT

TCTTTCGTTCCAGAAAGTCTTCGGTAAGTTCATCTCTTTCGCCCTTGAAATTAGTGAAGCTTTATCAG

CTTGCGTCATCGTGACATAAAACCTAGAAGAATCCAGATCAACCATAGTCACACTTTCGACAGCACGA

GATGTCACAGATCTCTTCACTATAGATTGAGAAACCCATCTGGTGTATTTTGAGTGGGTTCCTTTCGG

AAGGGCCTTCGTAGCCATTAAACGCCACCAAGCGGAGCCATTA
```

Fig. 1. Gene sequence of virus isolated from *Lagenaria siceraria*

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Query  17    GACTTC-GACGGTTTGTAGATTACCGCCAACTCAGGTCCCGCCTAAAGGAGGGACTCCGC  75
             ||||||  ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  3127  GACTTCAGACGGTTTGTAGATTACCGCCAACTCAGGTCCCGCCTAAAAGAGGGACTCCGC  3068

Query  76    TAAGCACGAGCAATACATTCGGCGATTAAGTCGCCGCCTAACATACCACAATACTCATAG  135
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3067  TAAGCACGAGCAATACATTCGGCGATTAAGTCGCCGCCTAACATACCACAATACTCATAG  3008

Query  136   CGGAAGGTGACCTTATGTCTAGTCAAAGCGACAAGACAATAAGAAAATTTCTTAAATAGG  195
             |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3007  CGGAAAGTGACCTTATGTCTAGTCAAAGCGACAAGACAATAAGAAAATTTCTTAAATAGG  2948

Query  196   TCACACTTTGTGCTCTTTAATCTTACCAAAGTAACGTGGTCCTCAGAAATACCCTGGGAC  255
             ||||||||||||||||||  |||||||||||||||||||||||||||||||||  ||||||
Sbjct  2947  TCACACTTTGTGCTCTTTAGTCTTACCAAAGTAACGTGGTCCTCAGAAATACCTTGGGAC  2888

Query  256   TCATGCACAGTTTTGATTCTTTCGTTCCAGAAAGTCTTCGGTAAGTTCATCTCTTTCGCC  315
             ||||||||||||||||||||||  |||||||||||||||||||||||||||||||||||||
Sbjct  2887  TCATGCACAGTTTTGATTCTTTCATTCCAGAAAGTCTTCGGTAAGTTCATCTCTTTCGCC  2828

Query  316   CTTGAAATTAGTGAAGCTTTATCAGCTTGCGTCATCGTGACATAAAACCTAGAAGAATCC  375
             ||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2827  CTTGAAATCAGTGAAGCTTTATCAGCTTGCGTCATCGTGACATAAAACCTAGAAGAATCC  2768

Query  376   AGATCAACCATAGTCACACTTTCGACAGCACGAGATGTCACAGATCTCTTCACTATAGAT  435
             ||||||||||  ||||||||||  |||||||||||||||||||||||||||||||  |||||
Sbjct  2767  AGATCAACCAGAGTCACACTTGCGACAGCACGAGATGTCACAGATCTCTTCACTTTAGAT  2708

Query  436   TGAGAAACCCATCTGGTGTATTTTGAGTGGGTTCCTTTCGGAAGGGCCTTCGTAGCCATT  495
             ||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  2707  TGAGAAACCCATCTGGTGTATTTTGAATGGGTTCCTTTCGGAAGGGCCTTCGTAGCCATC  2648

Query  496   AAACGCCACCAAGCGG  511
             ||||||   |||||||
Sbjct  2647  AAACGC--ACAAGCGG  2634
```

Fig. 2. Gene alignment of the virus sequence showing 97% sequence identity with *Cucumber mosaic virus* isolate, Sequence ID: MG021460.1

Query stands for virus sequence of *Lagenaria siceraria* while Subject is the *Cucumber mosaic virus* available at NCBI database.

4. DISCUSSION

4.1 Serological Tests

ACP-ELISA has been employed in the detection and identification of plant viruses into the genus taxon [23-29]. The detection of the virus isolate in this study by the cucumovirus antiserum suggests it belongs to the genus *Cucumovirus.*

4.2 Gene Sequence and Sequence Alignment

Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times [30-41].

A virus identity will become unassailable if the degree of homologue of it sequence is established after comparison with sequences of previously characterised members of the genus to which the virus in question belongs.

It has been suggested by [42,40,43] that virus sequence with less than approximately 76% sequence identity should be regarded as belonging to different species while isolates with 76-89% sequence identity should be considered as virus of the same strains and sequence presenting 90-100% sequence identity should be regarded as same virus.

The virus isolate in this study had sequence homologue of 97% with CMV which is above 90% and should therefore be considered as CMV. This is the first report of *Cucumber mosaic virus* infecting *L. siceraria* in Nigeria.

5. CONCLUSION

In conclusion, the virus under study was serologically confirmed to be a *Cucumovirus* and molecularly identified to be *Cucumber mosaic virus.* This is the first report of CMV in Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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