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First report of *Algerian watermelon mosaic virus* infecting *Cucumis sativus* L in South South Nigeria

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Abstract: *Cucumis sativus L is a member of the cucurbit family grown wildly for it edible fruits. Virus infection has been reported to be a major constraint to its production as over 60 plant viruses are known to infect this crop reducing the quality and quantity of yearly production. Symptoms of mosaic and mottle was observed on this crop during the 2019 growing season in Ehom, Cross River State, Nigeria. Infected leaf samples were collected and tested using host rang/symptomatology test, insect transmission test and gene sequence analysis. The virus was found to infect only members of the cucurbit family and was transmitted by Aphis spiraecola. A. citricida did not transmit the virus. ACP-ELISA detected the virus to be a potyvirus and result from gene sequence analysis showed that the virus shared 81 % sequence identity with Algerian watermelon mosaic virus. The virus was considered a strain of Algerian watermelon mosaic virus. This is the first report of this virus infecting Cucumis sativus in Nigeria.*

Background: *Cucumis sativus L is a vegetable crop belonging to cucurbit family. This crop has served as a source of income and food for local farmers in Ehom, Cross River State. It has been reported to contain phytonutrients which play important role in maintaining health. However, plant virus infection has been reported to reduce quantity and quality of production. Over 60 plant viruses are known to infect this crop and many other plants in the cucurbit family. A visit to some farms in this region prescribed evidence of viral infection on inspected gardens. Although several viruses infecting cucurbits have been isolated, characterised and identified in Nigeria there is little information on Algerian watermelon mosaic virus infection of crops in Nigeria.*

Material and Methods

Infected leaf sample of C. pepo were collected and tested using host range/symptomatology test, insect transmission study, ACP-ELISA test and gene sequence analysis.

Results: *The virus was found to infect only members of the cucurbit family and was transmitted by Aphis spiraecola. A. citricida did not transmit the virus. ACP-ELISA detected the virus to be a potyvirus and result from gene sequence analysis showed that the virus shared 81 % sequence identity with Algerian watermelon mosaic virus.*

Conclusion: *Through host range/symptomatology study, insect transmission test, serology and gene sequence analysis a strain of Algerian watermelon mosaic virus has been detected and found to infect C. sativus in Nigeria.*

Key Word: *Potyvirus, ACP-ELISA, Cucumis sativus, Aphis sequence*

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I. Introduction

Cucumis sativus L is one of the most economically important fruit bearing vegetables crops grown in the South Eastern part of Nigeria. The crop is believed to have originated from the Himalayas, possibly in India. It is a warm season, vining, annual plant in the Cucurbitaceae family grown for it fruits . Cucumbers as they are commonly called have become a source of income and most economically important crops that are grown both in commercially and small scale by local farmers, in addition to their role in local consumption. The increase of area devoted to cucurbit crops and the intensification of production has led to the emergence of severe viral epidemics that threaten the sustainability of the cultures[5].

Algerian watermelon mosaic virus (AWMV) belongs to the genus *Potyvirus* in the family *Potyviridae*. It has flexuous filamentous particles. According to reports the virus can cause economically important losses in the quality and quantity of several horticultural crops, mostly cucurbits. The geographical distribution of the

virus is not systematical and it depends on climatic conditions. AWMV was detected most commonly in temperate climate and subtropic regions. At least 29 species of Aphids, including *Myzus persicae* (Sulzer) and *Aphis craccivora* Koch, transmit the virus in a non-persistent manner[9]. It is also transmitted by mechanical inoculation on more than 170 plant species belonging to 27 families[25].

AWMV and other viruses are ranked among the most common causal agents of cucurbit diseases worldwide[21]. At least 60 viruses can infect plants in the *Cucurbitaceae* family, and new virus species on these hosts are described every year[21,17,22]. Cucumber mosaic virus (CMV), zucchini yellow mosaic virus (ZYMV), papaya ringspot virus (PRSV), and watermelon mosaic virus (WMV) are the most frequent and economically important viruses on a worldwide basis[17]**.** Few of these viruses have been characterized and identified in different part of Nigeria. For examples, Atiri[4] have characterised CMV in northern Nigeria. Alegbanjo, et al.[2] have characterize TMV in Northern Nigeria. Ayo-John, et al.[5] have further provided a checklist of viruses prevalence in south west of Nigeria. Eyong et al.[10] have also reported a new species of virus called *Telfairia severe mosaic virus* in Calabar Nigeria.

A visit to some of the farms during the 2019 planting season prescribed evidence of virus infection evident in mosaic and mottling symptoms which resulted to poor growth and yield losses. Reports on viruses infecting cucumber in South East Nigeria is lacking therefore this research is aimed at employing biological, serological and molecular tool in identifying the causal agent of cucumber disease in this region.

II. Material and methods

Sources and isolation of viruses

Symptomatic leaf tissues of *Cucumis sativus* revealing virus-like symptoms of mosaic were obtained from Ehom in Biase, Cross River State Nigeria and collected into Ziploc air tight polyethylene bags to keep the leaves fresh and ensure the viability of the virus. The virus was thereafter maintained on young seedlings of *Cucumeropsis mannii* through mechanical inoculation by triturating the symptomatic virus infected leaf tissues in pre-sterilized cold mortar and pestle in inoculation phosphate buffer of 8.0 pH and 0.03 M.

Collection of seeds and host range studies

Viable seeds used for host range studies were obtained from the wild and local farmers from Adim and Abini in Cross River State. They were 32 plant species belonging to 10 plant families tested. The seeds were sown in 16 cm diameter polyethylene bags, ¾ filled with sterilized humus soil at the rate of five seeds per bag. Inocula prepared from the symptomatic leaf tissues were inoculated mechanically on carborundum (600 mesh) dusted leaves of the test plants. The cucurbits and fabaceous plants were inoculated at the 2 leaf stage and others at 4-5 leaf stage. Inoculated leaves were then washed with water and left for symptoms development.

Insect transmission tests

Aphis spiraecola and *A. citricida* are two aphid species which ability to transmit the virus isolate used in this study was tested. The aphids were obtained from their natural host *Chromolaena odorata* and *Citrus sinensis* respectively. The insects were collected in a transparent plastic container over which a piece of gauze was use secure them. The insects were starved for one hour and then allowed acquisition access feeding period of 2-5 minutes on virus infected leaves. A paint brush was used to transfer 5 aphids to young seedlings of *Cucumeropsis manni,* (The test plant) inside insect proof screen cages for inoculation access feeding of 10 minutes. The aphids were killed by spraying them with aphicide (Pirimor).

Serological tests

Antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) as described by Kumar[15] was used for the detection of the potyvvirus. The infected leaf sample of *C. sativus* of 0.1g was triturated in 1mL of coating buffer (0.015M $Na_2 Co_3$ + 0.0349M $NaHCo_3$ + dH_2O) and dispensed into each well of ELISA plate. After incubation at 37°C for 1h the plate was washed 3 times with PBS-Tween for 3 min between each wash. Cross adsorption was made by grinding 1 g of healthy plant sample in 20mL 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 100µL of each antiserum polyclonal antisera was added to wells PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP + 0.02g $NaNO_3$) was added per well and the plates incubated at 37°C for 1h. The plates were again washed 3 times with PBS-T. One hundred-µL of $0.001g{\cdot}mL^{-1}$ of the ELISA plates and again incubated at 37°C for 1h. The ELISA plates were then washed 3 times with PBS-T. One hundred-µL of protein, A-alkaline phosphatase conjugate diluted in the ratio 1:15000 in conjugate buffer (1/2 of *p*-nitrophenyl phosphate substrate in substrate buffer (97mL diethanolamine + 800mL H_2O + 0.2g $NaNO_3$ and HCl to give pH 9.8) was added per well and incubated at room temperature for 1 h. For all incubations, plates were covered with ELISA cover plates to avoid edge effects and to maintain uniform temperature. Healthy plant sample was used as controls. After 1 h absorbance was measured at A_{405nm} using an

ELISA plate reader (Micro Read 1000 ELISA Plate Analyser, U.S.A) after 1 h of incubation. The samples were considered positive when the ELISA reading was at least twice the reading for the healthy control[15].

RNA extraction from infected leaf samples

Total RNA was obtained from fresh leaves of *C. sativus* using the cetyltrimethylammonium bromide (CTAB) protocol as described by Abarshi et al.[1]. One hundred milligrams of the infected leaf sample was grounded in sterile mortar and pestle in 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 use. Each of the homogenates 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 allowed to cool to room temperature. Then 0.75 ml of phenol choloform isoamyl (25:24:1) was added to each tube containing the homogenate. Each tube was then vortexed vigorously to form an emulsion and then centrifuged at the 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 the mixture was kept at -80^{o}C for 10 minutes. The mixture was centrifuged 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 centrifuged at 12,000 rcf for 5-10 minutes. The supernatant 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 reversed transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extracts from the leaves of healthy plants were used as negative control.

Reverse Transcriptase Polymerase Chain reaction (RT-PCR)

Virus-specific complementary DNA (cDNA) fragments were amplified from total nucleic acid derived from the infected leaf samples by a RT-PCR method as described by Pappu et al.[20]. The RT-PCR was performed using the cylindrical inclusion (CI) primers forward 5$^{'}$- GGIVVIGTIGGIWSIAARTCIAC-3$^{'}$, Reverse5$^{'}$-ACICCRTTYTCDATDATRTTIGTIGC-3$^{'}$ and CMV primers Forward 5'-TGGTCGTCCAACTATTAACCAC-3' Reverse 5'-TACTGATAAACCAGTACCGGTGA 3' as described by Ha et al.[12]. 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) and nucleic acid from infected sample (1:10 dilution) (3.0 µl).

Amplifications were carried out in a GeneAmp 9700 PCR system thermalcycler (Applied Biosystem Inc., USA) using the following thermocyclic conditions; 42^{o} C for 30 min for reverse transcription, 94^{o} C for 3 min for initial denaturing, followed by 40 cycles of denaturing at 94^{o} C for 30 sec, an annealing step at 40^{o} C for 30 s, an extension at 68^{o} C for 1 min and a final extension at 72^{o} C for 10 min 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 700bp).

Amplicon purification and sequencing

The RT-PCR amplicon for each sample was purified by adding 95 % ethanol to 40 µl of the amplicon in a new 1500 µl Ependorff tube and the solution was kept in – 80^{o} C for 10 minutes. The tube was centrifuged for 10 min and the supernatant discarded. Five hundred of 70 % ethanol was added and centrifuged at maximum speed for 5 min. The supernatant was discarded and the tube was left at room temperature to dry after which the purified cDNA was dissolved in 30 µl of sterile distill water. Sequencing was done by using automated DNA sequencer (Applied Biosystems ABI310) at International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria.

Sequence analysis

The virus identity under study was established after comparing sequence of the virus with known virus sequences in the GenBank available at National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLASTn) program[6].

III. Results

Host range/symptomatology study

Result of the host range/symptomatology study revealed that all members of the cucurbit family reacted to the virus. Other plant species were not susceptible to the virus. The cucurbits that showed symptoms included *Cucurbita moschata, Cucumis melo, Cucumis sativus, Lagenaria siceraria, Cucurbita pepo,*

Cucupmeropsis manni and *Trichosanthes cucumerina*, symptoms induced by the virus ranged from mosaic, mottling, leaf malformation/deformation, to rugosity. (Table 1 and Figures 1-7).

Table 1: Symptoms observed on assay plants through mechanical transmission using the sap of virus infected isolate

Plant species	Symptoms
Cucumis sativus	LMD
Cucupmeropsis manni	R
Cucurbita pepo	R
Cucurbita moschata	M
Luffa cylindrical	E
Lagenaria siceraria	LR
Trichosanthes cucumerina	LMD/Mo

M=Mosaic; M=Mosaic; Mo=Mottling; LMD=leaf malformation/deformation; R= Rugosity and LR- Leaf reduction

Figures 1: ***Cucumis sativus,*** **2:** ***Cucupmeropsis manni*** **3:** ***Lagenaria siceraria,*** **4:** ***Cucurbita moschata,*** **5:** ***Trichosanthes cucumerina,*** **6:** ***Luffa cylindrical,*** **7:** ***Cucurbita pepo***

Insect transmission tests

The insect transmission test results obtained showed that the virus was efficiently transmitted by *A. Spiraecola* with an average transmitted rate of 60 % when five aphids were used on the test plants. The plants revealed mosaic, mottle and leaf reduction. *Aphid citricida* did not transmit the virus.

Serology testing against universal potyvirus and cucumovirus antisera

In this test, the results obtained revealed positive reaction against the universal potyvirus antiserum. Reaction against the cucumovirus antiserum was negative. The optical density reading for the potyvirus at A405nm was 1.123 which is twice greater than 0.405 the absorbance from healthy controls (Table 2).

TABLE 2: Antigen Coated Plate (ACP) Enzyme Linked Immunosorbent Assay (ELISA) for detection of Cucumovirus and Potyviruses

Sample	**Location**	**OD reading at A_{405nm} against virus polyclonal antibodies**	
	Ehom	Cucumovirus	Potyvirus
C. pepo virus isolate		0.529	1.123*
Healthy control		0.326	0.405

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

Gene sequence and sequence alignment

Result for the nucleotide sequence for the *C. sativus* virus isolate is presented in Figure 8. Figure 9 represents the gene alignment of the sequence with other viruses available in the GenBank. The sequence analysis revealed 81 % sequence homologue with *Algerian watermelon mosaic virus*.

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TCGGTGGGTGGGGGTCGGATGCACAGGGGGGGGGTGCATCACCTGCTTTGCCAGTAAGTTCATGTTCTTTTGCTT
GAGGCCGGGCGATCTCTGTGCGAAAATGTATGTAATCCATTACGTGGGGATCCATTTAATCAAAATCCAACAATC
CGCATGCGTGGTATGACTTCGTTTGGATCATCCCCCATAACCATCATGACAAGTGGCTTCGCACTACACTATTTC
GCACACAGCGTGGAACAGCTTCAAGAGTTCGATTTCATAATCATTGACGAATGTCATGTCGTAGATGCTCAAGCG
ATGGGATTCTATTGCCTAGCCCATGAACACAAAATCAGTGGAAAGATTCTAAAGGTGTCGGCAACTCCGCCTGGA
AGAGAAACTGAGTTCACAACTCAGTTCCCGGTCAAGCTGGTGACAGAGGATCATATAAGCTTTCAACAACTTGTC
AACAACTTTGGGAGCGGGGCGAATAGTGATGTCACGACTGAAGCAGATAACATCCTTATTTATGTGGCAAGCTAT
AATGAAGTCGACCAGCTCGGCAATATGCTTAACGAAAAGGGATACAGAGTGACGAAAATTGATGGGCGAACAATG
AAGATCGGCAAGACAGAAATCACAAACGTATGGACACAAATGGACAAGAAGCACTTCATTGTTGCCACCAACATC
ATCGAAAAACGGCGTAAGA
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Fig 8: Gene sequence of *C. sativus* virus isolate

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Query   50      GCCAGTAAGTTCATGTTCTTTTGCTTGAGGCCGGGCGATCTCTGTGCGAAAATGTATGTA
109

            ||||| ||| | |||| ||| | |||||| |    ||| |  |||| ||||| ||||| |
Sbjct   4185    GCCAGAAAGGTAATGTCCTTCTACTTGAGCCAACACGACCATTGTGTGAAAACGTATGCA
4244
Query   110     ATCCATTACGTGGGGATCCATTTAATCAAAATCCAACAATCCGCATGCGTGGTATGACTT
169

            | | | | || ||||| ||||| |||||||||||| ||||| |||||||||||| ||||| |
Sbjct   4245    AACAACTGCGAGGGGACCCATTCAATCAAAATCCCACAATTCGCATGCGTGGAATGACAT
4304
Query   170     CGTTTGGATCATCCCCCATAACCATCATGACAAGTGGCTTCGCACTACACTATTTCGCAC
229

            | ||||| ||||||||  | || || ||||| ||||| || || |||||||| |||||||
Sbjct   4305    CATTTGGTTCATCCCCTGTGACTATTATGACGAGTGGTTTTGCGCTACACTACTTCGCAC
4364

Query   230     ACAGCGTGGAACAGCTTCAAGAGTTCGATTTCATAATCATTGACGAATGTCATGTCGTAG
289

            | |  ||||| |||||||| ||||| |||||||||||||||| |||||||| ||  |||
Sbjct   4365    ATAATGTGGATCAGCTTCAGGAGTTTGATTTCATAATCATTGATGAATGTCACGTTATAG
4424
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Query   290     ATGCTCAAGCGATGGGATTCTATTGCCTAGCCCATGAACACAAAATCAGTGGAAAGATTC
349
            |||| |||||||||||||| || |||||||| || |||||||| ||||| ||||| ||||
Sbjct   4425    ATGCCCAAGCGATGGGATTTTACTGCCTAGCTCACGAACACAAGATCAGAGGAAAAATTC
4484

Query   350     TAAAGGTGTCGGCAACTCCGCCTGGAAGAGAAACTGAGTTCACAACTCAGTTCCCGGTCA
409
            | ||||| || |||||||||||||| |||||||||| |||||||||||||| || || |
Sbjct   4485    TCAAGGTCTCAGCAACTCCGCCTGGGAGAGAAACTGAATTCACAACTCAGTTTCCAGTTA
4544

Query   410     AGCTGGTGACAGAGGATCATATAAGCTTTCAACAACTTGTCAACAACTTTGGGAGCGGGG
469
            |||| ||||| || |||||||| ||||| || ||||| |||||||||||||| || || |
Sbjct   4545    AGCTAGTGACTGAAGATCATATCAGCTTCCAGCAACTCGTCAACAACTTTGGAAGTGGAG
4604

Query   470     CGAATAGTGATGTCACGACTGAAGCAGATAACATCCTTATTTATGTGGCAAGCTATAATG
529
            | || ||||| |||||||  ||||| ||||| || || ||||| || || ||||| ||||
Sbjct   4605    CAAACAGTGACGTCACGAGCGAAGCGGATAATATTCTCATTTACGTCGCTAGCTACAATG
4664

Query   530     AAGTCGACCAGCTCGGCAATATGCTTAACGAAAAGGGATACAGAGTGACGAAAATTGATG
589
            |||||||||| || ||||||||| | |||||||||| || | |||||| ||| |||| |
Sbjct   4665    AAGTCGACCAACTAGGCAATATGTTGAACGAAAAGGGTTATACAGTGACAAAAGTTGACG
4724

Query   590     GGCGAACAATGAAGATCGGCAAGACAGAAATCACAAACGTATGGACACAAATGGACAAGA
649
            | || |||||||| || |||||||||||||  || ||| ||||| || ||  || ||||
Sbjct   4725    GACGCACAATGAAAATTGGCAAGACAGAAAT-TCAGACGCATGGA-ACTAA-AGATAAGA
478
Query  650   AGCACTTCATTGTTGCCACCAACATCATCGAAAA  683
              |||| |||||||||| || || || || || ||
Sbjct  4782  AGCATTTCATTGTTGCTACYAAYATYATTGARAA  4815
```

Query – *Cucurbita pepo* virus isolate sequence
Sbjct – *Algerian watermelon mosaic virus*

FIG 9: Gene alignment of C. sativus virus isolate from Ehom showing 81 % sequence identity with *Algerian watermelon mosaic virus* (EU410442)

IV. Discussion

The result of the host range studies indicated that this virus had a rather narrow host range causing systemic infection on all cucurbits tested with symptoms ranging from mosaic to rugosity. Other plant species tested did not show symptoms. Reports have shown that members of the genus *Potyvirus* to which this virus belong usually infect members of the cucurbit family[18,16,8,3]. This suggest that the virus in this study could belong to the genus *Potyvirus*.

Results obtained from this study showed that *A. spiraecola* successfully transmitted the virus in a stylet borne manner. *Aphid citricida* did not transmit the virus. Reports have implicated *A. spiraecola* in the transmission of viruses in the potyvirus genus and this result is consistent with aphids transmission of viruses in the genus *Potyvirus* which have been reported to be transmitted by aphids in a stylet borne manner. Similar observations have been reported for members of this genus and these viruses could obviously belong to the genus *potyvirus*[13,19].

ACP-ELISA has been employed in the detection and identification of plant viruses into the genus taxon[7,23,28,27,29]. The detection of the virus isolates in this study by universal potyvirus antiserum further suggests this virus belong to the genus *Potyvirus.*

Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times[8,14,26]. 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.

A standard criteria for categorising viruses was put forward by Shukla and Ward[24], King et al.[14] and Frenkel et al.[11]. They suggested that virus sequences with less than approximately 76 % sequence identity should be regarded as belonging to different species while sequence showing 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 in this study revealed sequence identity of 81 % falling between the thresh hold of 76-89 % and is therefore considered a strain of *Algerian watermelon mosaic virus*. This is the first report of AWMV infecting *C. Sativus* in Nigeria

V. Conclusion

Through host range/symptomatology study, insect transmission test, serology and gene sequence analysis a strain of *Algerian watermelon mosaic virus* has been detected and found to infect *C. sativus* in Nigeria.

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