Original Research Article

Molecular detection of a plant virus infection *Cucurbita* pepo L. in Obubra Local Government Area of Cross River State, Nigeria.

ABSTRACT

Cucurbita pepo L is a member of Cucurbitaceae family in the genus *Cucurbita*. It is grown in Nigeria as a leafy vegetable and for its edible seeds and fruits. This crop has been reported to play important role in nutrition and medicine. Mosaic and chlorotic spots were observed on leaves of *C. pepo* and obtained from the staff quarters of University of Calabar in 2021 planting season. This study was initiated to identify the virus causing infection on *C. pepo*. The diagnostic tool employed was RT-PCR and gene sequence analysis. The result obtained by RT-PCR with cylindrical inclusion (CI) primer showed a fragment of predicted size 700 bp. Sequence alignment and analysis showed that the virus isolate has 86% sequence identity with *Algerian watermelon mosaic virus* (EU410442.1) confirming that the virus under study is a strain of *Algerian watermelon mosaic virus.* This is the first report of *Algerian watermelon mosaic virus* in Nigeria.

Keyword: *Cucurbita pepo*, Mosaic, sequence, RT-PCR, cylindrical inclusion

INTRODUCTION

Cucurbita pepo L is an annual, monoecious climber cultivated worldwide for its edible fruits. This crop is a member of Cucurbitaceae family in the genus *Cucurbita*. The stems are fleshy, stout, setose and develop series of multifid tendrils. The leaves are simple. The petiole is stout, fleshy, setose and up to 10 cm long. The blade is triangular, 20–30 cm across, irregularly five-lobed, setose, cordate at base, dentate at base and acute at apex. The flowers are massive and solitary on a 2–20-cm-long, setose pedicel. The corolla is orangish, membranaceous, infundibuliform and five-lobed and can grow up to 10 cm long. In male flowers the calyx is campanulate and develops five linear segments which are 1–2.5 cm long. The androecium includes three stamens with 1.5-cm-long filaments. Female flowers

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present a reduced calyx and an ovary which is ovoid and unilocular. The fruiting pedicel is robust and pentagonal. The fruit is a berry which has a multitude of shapes and colors according to the varieties. The seeds are ovoid, flattened, 1–1.5 cm × 0.5–1 cm, white and smooth [21]

This crop is native to the America (originating from northeastern Mexico and Texas), where it has been cultivated for several thousand years [21]. Pumpkins were dispersed to other countries by transoceanic voyagers at the turn of the 16th century. The image *Quegourdes de turquie* (completed no later than 1508) represents the earliest-known representation of *C. pepo* in Europe. Native Americans dried strips of pumpkin and wove them into mats. They also roasted long strips of pumpkin and ate them. Galen, Hippocrates, Plinius, and Dioscorides used pumpkin seeds in form of compresses against swelling. Later, pumpkin was used for management of nephritis, tuberculosis, and internal worms and parasites. A famous painting entitled *Fruittivendola* (*The Fruit Seller*), located in Milan and painted in 1580, depicts the flower buds of *C. pepo* which were used for culinary purposes [8].

C. pepo is assumed to be a treasure house of antioxidants, polyunsaturated fatty acids (PUFA) and fibers which are known to have hepatoprotective and antiatherogenic properties [14]. In most countries of the world it is vigorously used in diabetes where it is used internally and superficially for treatment of worms and parasites. Pumpkin is also rich in oleic acid, linoleic acid, and tocopherols and has very high oxidative stability (Wasylikowa and van der Veen, 2004). Linoleic acid, a PUFA present in pumpkin seed oil, is known to increase membrane fluidity and allows for osmosis, intracellular, and extracellular gaseous exchange (Lovejoy, 2002). Pumpkin oil may play an important role in the protection against alcohol-induced hepatotoxicity and oxidative stress. Pretreatment with pumpkin oil may have hepatoprotective effects, which are varied and include oxidation, anti_lipid per oxidation enhanced detoxification, and protection against glutathione depletion [6].

Many factors decrease the quantity and quality of the production of this crop. Plant viruses are ranked the most common causal agent of diseases affecting this crop worldwide [19]. At least 60 viruses can infect plants in the Cucurbitaceae family, and new virus species on these hosts are described every year [19,12,20].

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C. pepo is widely cultivated in Nigeria including the South Eastern part of the country. This crop as well as other members of the cucurbit family are important vegetable crops worldwide but are subjected to more than 200 plant diseases [11]. Viruses rank among the most common causal agents of cucurbit diseases worldwide [19]. Cucurbits have been reported to be infected by as many as 60 plant viruses worldwide and many are yet to be reported [26,12]. Reports have shown that plant viruses have become a threat to production of this crop [16].

A visit to some farms and gardens in Calabar Municipality Local Government Areas of Cross River State, Nigeria in 2021 planting season revealed widespread infection of this crop with symptoms ranging from mosaic, chlorotic spots, mottling, leaf malformation/deformation, leaf reduction and stunting (Fig 1). The aim of this study is to isolate and characterize the virus causing infection on *C.* pepo in Obubra Local Government Areas of Cross River State with a view to identifying it.

Fig 1: Mosaic and chlorotic spot on *Cucurbita pepo*

MATERIALS AND METHODS

~~Sources~~ and isolation of viruses

Infected leaf sample from *C*. pepo with typical virus-like symptoms was collected from Obubra Local Government of Cross River State, Nigeria. This crop was found growing on farms and gardens in the location named above. Infected leaf samples were collected into Ziploc air tight polyethylene bags to keep them fresh to ensure the viability of the virus particles and later transported to the molecular laboratory of National Institute of Horticulture (NIHORT) Ibadan for the molecular diagnosis.

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177 **RNA extraction from infected leaf samples**

178 Total RNA was extracted from the infected leaf samples using the cetyltrimethylammonium bromide
179 (CTAB) protocol as described by [1]. One hundred milligrams of infected leaf sample was grounded in
180 sterile mortar and pestle in 1 ml extraction buffer (100 mM Tris-HCl, pH 8.0; 1.4 M NaCl; 20 mM
181 EDTA; 2 % CTAB) (hexadecetyltrimethylammonium bromide); and 0.4 % β- mercaptoethanol, added
182 just before use. Each of the homogenates was poured into a new 1.5 ml Eppendorf tube. The tubes
183 were vortexed briefly, incubated in a 60^{o}C water bath for 10 minutes and allowed to cool to room
184 temperature. Then 0.75 ml of phenol choloform isoamyl (25:24:1) was added to each tube containing
185 the homogenate. Each tube was then vortexed vigorously to form an emulsion and then centrifuged at
186 the speed of 12000 rcf for 10 minutes. The supernatant was then transferred to a clean 1.5 ml tube.
187 Three hundred of cold isopropanol was added to the supernatant to precipitate the nucleic acid (RNA)
188 and the mixture was kept at -80^{o}C for 10 minutes. The mixture was centrifuged at 12,000 rcf for 10
189 min to precipitate the nucleic acid. The supernatant was discarded and the nucleic acid pellet washed
190 in 500 µl of 70 % ethanol and centrifuged at 12,000 rcf for 5-10 minutes. The supernatant was
191 decanted and the resultant nucleic acid pellet was air-dried at room temperature. Nucleic acid pellet
192 was then re-suspended in 50 µl sterile distilled water and used as a template source for reversed
193 transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extracts from the leaves of healthy
194 plants were used as negative control.

195 **Reverse transcriptase polymerase chain reaction (RT-PCR)**

196 Virus-specific complementary DNA (cDNA) fragments were amplified from total nucleic acid derived
197 from the infected leaf samples by a RT-PCR method as described by [18]. RT-PCR was performed
198 using the cylindrical inclusion (CI) primers forward 5'- GGIVVIGTIGGIWSIAARTCIAC-3', Reverse 5'-
199 ACICCRTTYTCDATDATRTTIGTIGC-3' as described by [9]. The RT-PCR reaction mixture (50 µl)
200 consisted of I µl each of CICP 5' and CICP 3', 5x Go Taq green buffer (10.0 µl), $MgCl_2$ (3.0), dNTPs
201 (1.0 µl), Reverse transcriptase (0.24 µl), Taq DNA polymerase (Promega) (0.24 µl), sterile distilled
202 water (30.52 µl) and nucleic acid from infected leaf sample (1:10 dilution) (3.0 µl).
203

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.

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~~distil water. The product was sequenced at Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA) Ibadan.

Sequence analysis

The sequence identity between the virus under study was established by comparison with known virus sequences in the GenBank available at National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence identities were calculated from the sequence identity matrix option in MEGA 6 window software.

RESULTS

Reverse transcriptase polymerase chain reaction (RT-PCR)

The result obtained by RT-PCR with cylindrical inclusion (CI) primer showed a fragment of predicted size 700 bp. Sequence alignment and analysis showed that the virus isolate has 86% sequence identity with *Algerian watermelon mosaic virus* (EU410442.1) (Fig 2 and 3).

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CGGGTGGTTGGGGGGCGGGGAAATCGACTGGCTTACAACGTTTACGTAGCCGTGAGGGGGAGTGTTCTTCTACTT
GAACCCACTCGACCTCTGTGTGAAAATGTATGCAAGCAACTGCGAGGGGACCCATTTAATCAAAACCCAACAATC
CGTATGCGTGGAATGACATCGTTTGGTTCATCCCCCATAACCATTATGACGAGTGGGTTTGCGCTGCACTATTTC
GCACACAACGTGGATCAACTTCAGGAATTTGATTTCATAATCATTGATGAATGTCACGTCATAGACGCTCAAGCA
ATGGGACTTTACTGCCTGGCCCACGAACATAAGATCAGAGGGAAAATTCTCAAGGTTTCAGCAACTCCACCTGGG
AGAGAAACTGAGTTCACAACTCAGTTTCCAGTCAAGCTGGTGACGGAAGATCATATTAGCTTTCAGCAACTCGTT
AACAACTTTGGAAGTGGGGCGAACAGCGATGTTACAAGTGAAGCGGATAATATTCTCATTTACGTTGCGAGCTAT
AATGAAGTTGACCAACTAGGCAACATGCTGAATGAGAAGGGTTACAGAGTCACGAAGGTAGATGGACGCACAATG
AAAATTGGCAAAACAGAAATCCAAGACACATGGAACCAAGGATAAGAAACATTTCATTGTTGCCACCAACATCAT
CAAAAAACGGGCGTAA
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FIG 2: Gene sequence for *Cucurbita pepo* virus isolate

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Query  64 TGTTCTTCTACTTGAACCCACTCGACCTCTGTGTGAAAATGTATGCAAGCAACTGCGAGG 123
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Sbjct4198 TGTCCTTCTACTTGAGCCAACACGACCATTGTGTGAAAACGTATGCAAACAACTGCGAGG 4257
Query 124 GGACCCATTTAATCAAAACCCAACAATCCGTATGCGTGGAATGACATCGTTTGGTTCATC 183
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Sbjct4258 GGACCCATTCAATCAAAATCCCACAATTCGCATGCGTGGAATGACATCATTTGGTTCATC 4317

Query184  CCCCATAACCATTATGACGAGTGGGTTTGCGCTGCACTATTTCGCACACAACGTGGATCA 243
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Sbjct 4318CCCTGTGACTATTATGACGAGTGGTTTTGCGCTACACTACTTCGCACATAATGTGGATCA4377

Query244 ACTTCAGGAATTTGATTTCATAATCATTGATGAATGTCACGTCATAGACGCTCAAGCAAT 303
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Sbjct4378GCTTCAGGAGTTTGATTTCATAATCATTGATGAATGTCACGTTATAGATGCCCAAGCGAT4437

Query304  GGGACTTTACTGCCTGGCCCACGAACATAAGATCAGAGGGAAAATTCTCAAGGTTTCAGC 363
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Sbjct4438 GGGATTTTACTGCCTAGCTCACGAACACAAGATCAGAGGAAAAATTCTCAAGGTCTCAGC4497
Query364  AACTCCACCTGGGAGAGAAACTGAGTTCACAACTCAGTTTCCAGTCAAGCTGGTGACGGA 423
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Sbjct4498 AACTCCGCCTGGGAGAGAAACTGAATTCACAACTCAGTTTCCAGTTAAGCTAGTGACTGA4557
Query424  AGATCATATTAGCTTTCAGCAACTCGTTAACAACTTTGGAAGTGGGGCGAACAGCGATGT 483
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Sbjct4558 AGATCATATCAGCTTCCAGCAACTCGTCAACAACTTTGGAAGTGGAGCAAACAGTGACGT4617

Query484  TACAAGTGAAGCGGATAATATTCTCATTTACGTTGCGAGCTATAATGAAGTTGACCAACT 543
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Sbjct4618 CACGAGCGAAGCGGATAATATTCTCATTTACGTCGCTAGCTACAATGAAGTCGACCAACT4677

Query544  AGGCAACATGCTGAATGAGAAGGGTTACAGAGTCACGAAGGTAGATGGACGCACAATGAA 603
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Sbjct4678 AGGCAATATGTTGAACGAAAAGGGTTATACAGTGACAAAAGTTGACGGACGCACAATGAA4737

Query604  AATTGGCAAAACAGAAATCCAAACACATGGAACCAAGGAGTAAGAAACATTTCATTGTTG 663
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Sbjct4738 AATTGGCAAGACAGAAATTCAGACGCATGGAACTAAAGA-TAAGAAGCATTTCATTGTTG4796
Query  664   CCACCAACATCATAGAAAA  682
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Sbjct  4797  CTACYAAYATYATTGARAA  4815
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FIG 3: Gene alignment of *Cucurbita pepo* virus isolate showing 86 % sequence identity with *Algerian watermelon mosaic virus*

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DISCUSSION

Reverse transcriptase polymerase chain reaction (RT-PCR)

Induction of cytoplasmic cylindrical inclusions is a major characteristic of the members of the genus *Potyvirus* [2,17,]. The use of CI primers in PCR led to the production of amplicons of 700 bp, this is a proof that the virus isolate is a member of the genus *Potyvirus*. This test is similar to the detection of potyvirus using CI primers with the resultant production of amplicons of 700 bp [5].

Gene sequence and sequence alignment

Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times [15,10,24]. 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 [22,] [10], and [7] that virus sequences 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 in this study revealed sequence identity of 86 % falling between the thresh hold of 76-89 % and is therefore considered a strain of *Algerian watermelon mosaic virus*. Nigeria. Eyong et al. [3] and Eyong et al. [4] have characterised and identified viruses infecting some cucurbits in Cross River State using this same method.

CONCLUSION

A visit to some farms and gardens in the staff quarters of University of Calabar in 2021 revealed viral infection on *C. pepo.* Infected leaf sample with typical virus-like symptoms was collected and tested against RT-PCR using cylindrical inclusion (CI) primers, after which amplicons were sent for sequencing. Sequence was obtained and analysed. The result revealed 86 % sequence identity with *Algerian watermelon mosaic virus.* This is the first report of AWMV infecting *C. pepo* in this location.

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COMPETING INTERESTS

Authors have declared that there is not competing interest

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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