Annual Research & Review in Biology

35(11): 66-72, 2020; Article no.ARRB.57872
ISSN: 2347-565X, NLM ID: 101632869

Serological and Molecular Characterisation of Virus Infecting Watermelon (*Citrullus lanatus*) in Adim-Biase, Cross River State, Nigeria

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Authors' contributions

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

Article Information

DOI: 10.9734/ARRB/2020/v35i1130300
Editor(s):
(1) Dr. Jin-Zhi Zhang, Huazhong Agricultural University, China.
Reviewers:
(1) Filiz Randa Zelyüt, Ankara University, Turkey.
(2) Celile Aylin Oluk, Eastern Mediterranean Agriculture Research Institute, Turkey.
(3) Ravindra Kumar, Siddharth University, India.
Complete Peer review History: http://www.sdiarticle4.com/review-history/57872

Original Research Article

Received 08 April 2020
Accepted 12 June 2020
Published 14 November 2020

ABSTRACT

Watermelon, *Citrullus lanatus*, (Thunb.) is an annual crop in the Cucurbitaceae family grown for it fruits. It nutritional and medical uses helps in combating some illnesses and maintaining good health. The production of this crop in Nigeria is seriously under threat by virus disease. A survey of some farms in Adim, Biase Local Government Area of Cross River State, Nigeria during the 2019 planting season revealed mosaic and chlorotic symptoms. Mechanical inoculation was performed on young seedlings of *Cucumeropsis mannii* from watermelon samples showing viral symptoms and latter tested against the universal potyvirus antiserum and further detected by RT-PCR assay using cylindrical inclusion (CI) primer. The amplified cDNA was cloned and the nucleotide sequence determined. The serology result showed that there was positive reaction against the potyvirus antiserum confirming it to be a potyvirus. The gene sequence analysis revealed 86% sequence identity with *Algerian watermelon mosaic virus.* This is the first report of *Algerian watermelon mosaic virus* strain infecting watermelon in Nigeria.

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Keywords: ACP-ELISA; RT-PCR; watermelon; AWMV; Nigeria.

1. INTRODUCTION

Watermelon, *Citrullus lanatus*, (Thunb.) is a vining annual plant in the family Cucurbitaceae grown for its fruit. The crop is usually consumed as a fresh fruit. In Africa it is sometimes cooked before eating and may also be used as an animal feed. Watermelons have become synonymous with summer and picnics, and for good reason it has refreshing quality and sweet taste help to combat heat and provide a guilt-free, low maintenance dessert [1]. Because of its water and fibre contents, watermelon has been reported to help prevent constipation and promotes healthy digestive tract [2]. Studies have shown that watermelon is helpful in combating cancer as a result of its antioxidants properties [3]. Watermelon and watermelon juice have been shown to reduce muscle soreness and improve recovery time following exercise in athletes [4,5,6].

According to reports of Turkish Statistical Institute in 2009, the estimated annual production of vegetable crops is approximately 1,42 million tons covering approximately an area of 32,900 ha. Cucurbitaceae family constitutes about 70% of the crops grown. Watermelon ranks first (841,805 tons), followed by melon (103,765 tons), cucumber (20,890 tons) and squash (15,563 tons) in world cucurbit production [7].

Nigeria has a vast abundance of land, resources and climatic variations that enable it to grow different varieties of food and cash crops in its agricultural sector. As a nation with the most immerse Agricultural sector in Africa, the local agricultural industry employs up to 70% of the local workforce and contributes about 40% of the country's GDP. One of such agribusiness that contributes to the food and economic value of the country is Watermelon farming in Nigeria. According to Wikipedia, the worldwide production of watermelon as at 2014 was 111 million metric tonnes, with China accounting for 67% of the total production. This large production rate has influenced watermelon demand positively both locally and internationally, making it a lucrative agribusiness for local farmers to venture into. With a widespread demand for the crop, watermelon farming in Nigeria is growing at a fast pace.

Viral infection has become a major threat to the quantity and quality of production of this crop. Research has shown that over 60 viruses can infect plants in the Cucurbitaceae family, and new virus species on these hosts are described every year [8,9,10]. The most encountered of them include Cucumber mosaic virus (CMV, genus *Cucumovirus*), Zucchini yellow mosaic virus (ZYMV, genus *Potyvirus)*, Papaya ringspot virus (PRSV, genus *Potyvirus),* and Watermelon mosaic virus (WMV, genus *Potyvirus)* are the most frequent and economically important viruses on a worldwide basis [8]. Several of these viruses have been isolated and identified worldwide including Nigeria. For example, [11] has reported infection of fluted pumkin by CMV in northern Nigeria. [12] have also provided a checklist of viruses prevalent in south west of Nigeria. [13] have further reported infection of cucumber by PRSV and infection of *Cucurbita moschata* by *Morroccan watermelon mosaic virus* (MWMV) in Calabar Southern Nigeria [14]. [10] have also reported a new species of virus called *Telfairia severe mosaic virus* infecting *Telfairia occidentalis* and *Algerian watermelon mosaic virus* infecting cucumber [15] in Calabar. However, these studies have not been done extensively at serological and molecular level. Therefore this study is aimed at employing serological and molecular tools in identifying the virus responsible for infection of watermelon. This paper reports ACP-ELISA testing and sequence analysis of the gene of the watermelon virus isolate obtained from Adim in South South Nigeria.

2. MATERIALS AND METHODS

2.1 Virus Sources and Mechanical Inoculation

Three infected leaf samples of watermelon showing typical virus-like symptoms of mosaic and chlorotic spots were obtained from Adim, Biase Local Government Area of 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 five young seedlings of *Cucumeropsis mannii* through mechanical inoculation by triturating the symptomatic virus infected leaf tissues in pre-sterilized cold mortar and pestle using phosphate buffer of 8.0 pH and 0.03 M.

Fig. 1. Mosaic symptoms on *C. mannii* (The indicator plant used in maintaining the virus after isolation from the field)

2.2 Serological Tests

Antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) as described by [16] was used for the detection of potyvvirus. 0.1g of infected leaf sample of *Cucumeropsis mannii* which served as indicator plant 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 20 mL of conjugate buffer (1/2 PBS + 0.05% Tween 20 + 0.02% egg albumin + 0.2% PVP). The universal potyvirus antiserum was diluted at 1:3000 in the adsorption solution and 100 µL of the antiserum polyclonal antisera was added to wells of the ELISA plates and again incubated at 37°C for 1 h. 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 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 1h. The plates were again washed 3 times with PBS-T. One hundred-µL of 0.001 $g{\cdot}mL^{-1}$ 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 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 [16].

2.3 Rna Extraction from Infected Leaf Samples

Total nucleic acid was obtained from infected leaves of *Cucumeropsis mannii* which served as indicator plant using the cetyltrimethylammonium bromide (CTAB) protocol as described by [17]. 100 mg of each infected leaf sample was grounded in sterile mortar and pestle in 1 ml extraction buffer (100 mMTris-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 transferred into a new 1.5 ml sterile tube. The tubes were vortexed briefly, incubated in a 60°C water bath for 10 min and allowed to cool to room temperature. Then 750 µl of phenol-choloform-isoamyl alcohol (PCI) (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 g for 10 min. The supernatant was then transferred to a clean 1.5 ml tube. 300 µl of cold isopropanol was added to the supernatant to precipitate the nucleic acid and the mixture was kept at -80°C for 10 minutes. The mixture was centrifuged at 12,000 g 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 g for 5-10 min. 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 reverse transcriptase polymerase chain reaction (RT-PCR). Nucleic acid extracts from the leaves of healthy plants were used as negative control.

2.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Virus-specific complementary DNA (cDNA) were synthesized from total nucleic acid derived from the infected leaf of *Cucumeropsis mannii* used to maintain the virus isolated from watermelon by RT-PCR method as described by [18]. The RT-PCR was performed using the cylindrical inclusion (CI) primer.

Forward CICP 5' (5'-GGIVVIGTIGGIWSIAARTCIAC-3'), Reverse CICP 3' (5'-ACICCRTTYTCDATDATRTTIGTIGC-3') as described by [17]. The RT-PCR reaction mixture (50 µl) consisted of 1 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 µM), Taq DNA polymerase (Promega, USA) (0.24 µM), 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°C for 30 min for cDNA synthesis, 94°C for 3 min 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 min and a final extension at 72°C for 10 min ended the RT-PCR reaction. The PCR products were separated on 1.5% agarose gel, subsequently stained with ethidium bromide, visualized under UV light and photographed RT-PCR assay produced a PCR amplicon of expected size (approximately 700 bp).

2.5 Amplicon Purification and Sequencing

The RT-PCR amplicon for the infected sample was purified by adding 95% ethanol to 40 µl of the amplicon in a new 1500 µl steril tube and the solution was kept in – 80°C for 10 minutes. The tube was centrifuged for 10 min and the supernatant discarded. 500 µl 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 product was dissolved in 30 µl of sterile Distilled water. Sequencing was done by using Sanger sequencer (Applied Biosystems ABI310) at INQABA Biotec West Africa Ibadan, Nigeria.

2.6 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 [19].

3. RESULTS

The ACP-ELISA test result revealed a positive reaction against the universal potyvirus antiserum. The optical density reading for the potyvirus at A405nm was 1.885 which is twice greater than 0.405 the absorbance from the healthy controls (Table 1).

3.1 Gene Sequence Alignment and Analysis

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

4. DISCUSSION

In this study, a virus *Algerian watermelon mosaic virus* (AWMV) isolated from a naturally infected watermelon (*Citrullus lanatus* (Thunb.) plant exhibiting mosaic and chlorotic spot symptoms and was characterized serologically and molecularly. The serology test was performed using ACP-ELISA, the virus when inoculated on the indicator plant (*Cucumeropsis manni*) showed symptoms of mosaic and leaf malformation/deformation and further reacted positively against the universal potyvirus antiserum.

Table 1. Antigen coated plate (ACP) enzyme linked immunosorbent assay (ELISA) for detection of potyvirus

Sample	Location	OD reading at A_{405nm} against virus polyclonal antibodies
	Adim	Potyvirus
Watermelonvirus isolate		1.885*
Healthy control		0.405

**Sample was considered virus positive when the optical density (OD) reading at A405nm was 2x greater than the absorbance from healthy controls*

Score	**Expect**	**Identities**	**Gaps**
693 bits(768)	**0.0**	**515/600(86%)**	**5/600(0%)**

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Query  94    TGTGTGACGATGTATGTACTCTGCTGCTAGGGGACCCATTTAATCCAAACCCAACAATTC  153
             |||||||  | |||||  |  |  ||||  ||||||||||||  ||||  |||  ||  |||||||
Sbjct  4227  TGTGTGAAAACGTATGCAAACAACTGCGAGGGGACCCATTCAATCAAAATCCCACAATTC  4286

Query  154   GTATGCGTGTAATGAATTCGTTTGGTTCATCCCCCATAACCATTATGACTAGTGGGTTTG  213
             | |||||||  |||||  ||  ||||||||||||||  | ||  ||||||||  |||||  ||||
Sbjct  4287  GCATGCGTGGAATGACATCATTTGGTTCATCCCCTGTGACTATTATGACGAGTGGTTTTG  4346

Query  214   CGCTACACTATTTCGCACACAACGTGTATCAGCTTCAAGAATTTGATTTCATAGTCATTG  273
             ||||||||||  ||||||||  ||  |||  ||||||||||  ||  ||||||||||||  ||||||
Sbjct  4347  CGCTACACTACTTCGCACATAATGTGGATCAGCTTCAGGAGTTTGATTTCATAATCATTG  4406

Query  274   ATGAATGTCATGTTATAGATGCCCAAGCAATGGGATTTTATTGCCTAGCCCACGAACATA  333
             ||||||||||  ||||||||||||||||  ||||||||||  ||||||||  ||||||||  |
Sbjct  4407  ATGAATGTCACGTTATAGATGCCCAAGCGATGGGATTTTACTGCCTAGCTCACGAACACA  4466

Query  334   AGATCAGAGGGAAAATTCTCAAGGTTTCAGCAACTCCACCTGGGAGAGAAACTGAGTTCA  393
             ||||||||||  ||||||||||||||  ||||||||||  ||||||||||||||||||  |||||
Sbjct  4467  AGATCAGAGGAAAAATTCTCAAGGTCTCAGCAACTCCGCCTGGGAGAGAAACTGAATTCA  4526

Query  394   CAACTCAGTTTCCAGTCAAGCTGGTGACAGAAGATCATATTAGCTTTCAGCAACTCGTTA  453
             ||||||||||||||  |||||  |||||  ||||||||||  |||||  ||||||||||  |
Sbjct  4527  CAACTCAGTTTCCAGTTAAGCTAGTGACTGAAGATCATATCAGCTTCCAGCAACTCGTCA  4586

Query  454   ACAACTTTGGGAGTGGAGCAAACAGCGATGTCACAAGTGAAGCGGATAATATTCTCATTT  513
             ||||||||||  ||||||||||||||  ||  |||||  ||  ||||||||||||||||||||
Sbjct  4587  ACAACTTTGGAAGTGGAGCAAACAGTGACGTCACGAGCGAAGCGGATAATATTCTCATTT  4646

Query  514   ACGTCGCGAGTTATAATGAAGTTGACCAGCTAGGCAACATGCTGAATGAGAAGGGTTACA  573
             |||||||  ||  ||  ||||||||  |||||  ||||||||  |||  ||||  ||  ||||||||  |
Sbjct  4647  ACGTCGCTAGCTACAATGAAGTCGACCAACTAGGCAATATGTTGAACGAAAAGGGTTATA  4706

Query  574   GAGTCACAAAGGTTGATGGACGCACAATGAAAATTGGCAAAACTGAAATTCAAACATCAT  633
              |||  |||||  |||||  ||||||||||||||||||||||  ||  ||||||||  ||  |||
Sbjct  4707  CAGTGACAAAAGTTGACGGACGCACAATGAAAATTGGCAAGACAGAAATTCAGAC-GCAT  4765

Query  634   AGGAACTCAAGGATAAGGGAAACATTTCATCGTTGCCACCAACATCATCGAAAACGGCGT  693
             ||||||  ||  |||||  |||  ||||||||  |||||  ||  ||  ||  ||  ||  ||  ||  ||
Sbjct  4766  -GGAACT-AAAGATAA--GAAGCATTTCATTGTTGCTACYAAYATYATTGARAATGGAGT  4821
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Fig. 2. Sequence alignment of watermelon virus isolate from Adim showing 86 % percent identity with *Algerian watermelon mosaic virus* (EU410442.1)

Query – Gene sequence of watermelon virus isolate
Sbjct – Algerian watermelon mosaic virus

ACP-ELISA has been employed as a tool in the detection of virus into genus taxon [20,21,22,4]. The detection of the virus isolate in this study by universal potyvirus antiserum therefore confirms that the virus isolate is a Potyvirus. [10,15] have also employed ACP-ELISA in the detection of TeSMV and strain of AWMV which are potyviruses infecting *Telfairia occidentalis* and *Cucumis sativus* respectively.

It has been suggested by [23,24,25] 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 isolated from watermelon in this study had 86% sequence identity falling between the thresh hold of 76-89% and is however considered a strain of AWMV in Nigeria. [10,15,26,27] have reported the identification of plant viruses using these criteria. This is the first report of AWMV in Nigeria.

5. CONCLUSION

This study has provided information on the identity of a virus known as *Algerian watermelon mosaic virus* infecting watermelon in Nigeria. It has further provided information that will form the basis of sustainable management strategies against viruses and the diseases it causes on cucurbits in Nigeria. More importantly, it also provided a first step approach in developing a program for producing transgenic crops with resistance to multiple virus infections for farmers in Nigeria. This is of particular importance in terms of genetically variable virus strains being able to overcome strain-specific resistance in an introduced transgenic cultivar, hence nullifying considerable time consuming and costly efforts in resistance development.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/57872