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Occurrence, Distribution and Identification of Viruses Infecting Some Cucurbits Across Major Cucurbit-Growing Areas in 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, wrote the protocol and wrote the first draft of the manuscript. Authors EEE managed the analyses of the study. Author OAI read the first draft of the manuscript. All authors read and approved the final manuscript

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ABSTRACT

Cucurbits are group of plants which belonged in the Cucurbitaceae family and constitute one of the most important vegetables for human consumption throughout the world. A survey conducted between January to March 2021 across major cucurbit-growing area in Cross River State revealed wide spread virus infection of these crops, surveyed plants showed mosaic, mottle and leaf deformation symptoms. This research was aimed at identifying viruses infecting some cucurbits in the study areas. Twenty four samples showing virus like symptoms were collected and tested against ACP-ELISA and RT-PCR. Result obtained from ACP-ELISA showed that 20 out of 24 samples collected tested positive to universal potyvirus antisera, the gene sequence analysis revealed that samples were predominantly infected with Algerian watermelon mosaic virus representing 40% of total viruses detected followed by Morroccan watermelon mosaic virus representing 15% of total viruses detected followed by Papaya ringspot virus, Zucchini tigre mosaic virus, Watermelon mosaic virus and Soybean mosaic virus representing 10% each for total viruses detected and finally Potato virus Y representing 5% of total virus detected. This survey is an

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accurate and timely detection and will form the basis for mitigating yield losses resulting from virus infection on cucurbits. This is the first report of wide scale detection of viruses infecting cucurbits in Nigeria.

Keywords: Cucurbitaceae; ACP-ELISA; RT-PCR; potyvirus; antisera.

1. INTRODUCTION

Cucurbits represent one of the most important vegetables for human consumption throughout the world. Cucurbit species are members of the family Cucurbitaceae that roughly includes 120 genera and 800 species, which are predominantly tropical plants though some, such as four major cucurbit crops are economically important worldwide: melon (*Cucumis melo* L.), cucumber (*Cucumis sativus* L.), watermelon (*Citrullus lanatus* (Thumb.) Mat. & Nak.), squash and pumpkin (*Cucurbita* spp.) [1]. Global production of cucurbits cultivated in the world is 151,212,210 tons. China is the main producer with 83,429,409 tons of melon, watermelon, cucumber and squash, while the USA is the main producer in the Western Hemisphere with 4,602,370 tons [1]. Cucurbit crops are often 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), are also serious constraints to their cultivation [3,4,5]. Aphids, whiteflies and thrips can become serious limitations to cucurbit production through the extensive damage they provoke, but also through the transmission of viral disease [6,7,8]. At least 59 viruses infecting cucurbits are known [9], whose outbreaks have led to significant yield losses worldwide [6,10,11].

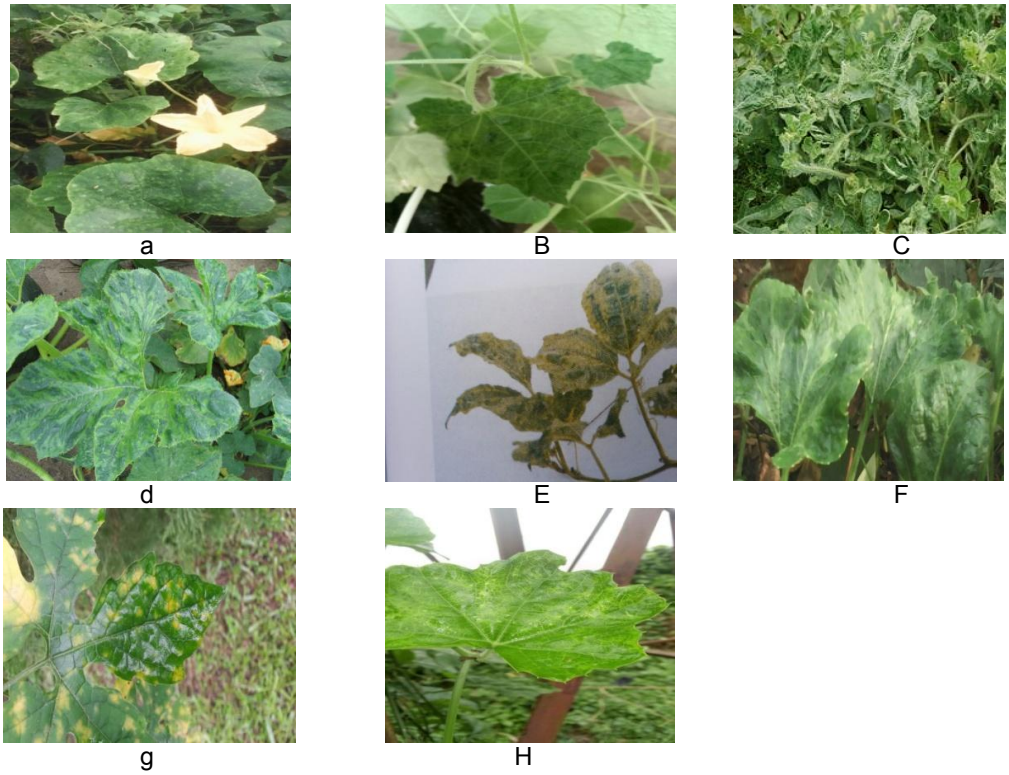

Fig. 1 (a-h). a- Mosaic and chlorotic spot on *Cucurbita pepo,* b-Rugosity and mottle on *Zeneria sp,* c- Leaf malformation and mosaic on watermelon, d- Mosaic, green vein banding and leaf malformation on cucumber, e- leaf malformation/deformation and chlorosis on *Telfairia occidental,* f-Mosaic and leaf deformation on *Cucurbita moschata,* g-Chlorotic spots on *Momordica charantia,* h-Mosaic on *Lagenaria siceraria*

The most commonly encountered of these viruses of cucurbits are *Papaya ringspot virus*-W (PRSV-W), *Squash leaf curl virus* (SLCuV), *Soybean mosaic virus* (SMV), *Squash mosaic virus* (SqMV) and *Squash vein yellowing virus* (SqVYV). Others include *Tobacco ringspot virus* (TRSV), *Watermelon mosaic virus* (WMV), *Watermelon silver mottle virus* (WSMoV), *Alfalfa mosaic virus* (AMV), *Bean pod mottle virus* (BPMV), *Cucurbit aphid borne yellows virus* (CABYV), *Cucurbit yellow stunting disorder virus* (CYSDV), *Cucumber green mottle mosaic virus* (CGMMV), *Cucumber mosaic virus* (CMV), *Melon necrotic spot virus* (MNSV), *Zucchini yellow mosaic virus* (ZYMV), and *Zucchini green mottle mosaic virus* (ZGMMV). These viruses have emerged as the most economically significant viruses infecting cucurbits in various cucurbit-growing regions of the globe [10]. Of these viruses CMV, SqMV, WMV-1 (now renamed WMV), WMV-2, ZYMV beside SqMV which is seedborne in melon are transmitted by beetles. Others are transmitted mechanically and by several aphid species in a non persistent manner [2]. Only very few of these viruses have been characterized and identified in Nigeria. For example, [12] have reported infection of fluted pumpkin by *Cucumber mosaic virus* in northern Nigeria, [13] have also characterized and reported *Yam mosaic virus* (YMV) and *Telfairia mosaic virus* (TeMV) infecting cucurbits in Northern Nigeria. [14] havefurther provided a checklist of viruses prevalent in south west of Nigeria [15] also reported infection of cucumber by *Papaya Ringspot virus* (PRSV) and infection of *Cucurbita moschata* by *Morroccan watermelon mosaic virus* (MWMV) in Calabar Southern Nigeria. However, Information regarding large scale infection of cucurbits in Southern Nigeria is lacking hence this study. A visit to some cucurbit growing farms in these areas between January to March 2021 revealed widespread infection of these crops. Infected plants exhibited a wide range of virus-like symptoms such as mosaic, chlorotic spots, mottling, leaf malformation/deformation and leaf reduction, some of which are presented in Fig. 1 (a-h). This research was therefore designed to carry out a survey on potyviruses infecting some cucurbits across different regions in Cross River State Nigeria, employing serological and molecular tools with a view to identifying them.

2. MATERIALS AND METHODS

2.1 Sources and Isolation of Viruses

Twenty-four (24) samples with typical virus-like symptoms were collected from different cucurbit crops, which included *Telfairia occidentalis*, *Cucumis sativus, Cucurbita pepo, Lagenaria siceraria, Citrullus lanatus*, *Zehneria spp, Cucumeropsis mannii, Momordica charantia* and *Cucurbita moschata*. These cucurbits were found growing on farms and gardens in Calabar, Baccocco, Akamkpa, Ehom, Akpet, Abini, Adim, Iwuru in Southern Ekori, Nko, Mkpani, Ofodua, Ofatura, Okuni, Ikom, Ugep in Central and Afrike, Alege, Igwo, Igoli, Okuku, Sankwala, Basang and Udeshi in Northern Cross River State, Nigeria. Infected leaf samples were collected into Ziploc air tight polyethylene bags to keep them fresh to ensure the viability of the viruses and later transported to the Molecular Laboratory of National Institute of Horticulture Ibadan, Nigeria for serology and molecular testing while the sequencing was done at Inqaba Biotech West Africa (IBWA), Ibadan, Nigeria.

2.2 Serological Tests

Antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) as described by [16] was used to determine the genus to which the virus isolates belonged. Symptomatic leaf samples of 0.1g were 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). Universal potyvirus and Cucumovirus antisera were diluted at 1:3000 in the adsorption solution and 100µL of each antiserum polyclonal antisera was added to wells of the ELISA plates and again incubated at 37°C for 1hour. The ELISA plates were then washed 3 times with 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 + 0.02g $NaNO_3$) was added per well and the plates incubated at 37°C for 1h. The plates were again washed 3 times with PBS-Tween. One hundred-µL of 0.001g·mL^{-1} 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 samples were 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 Total RNA Extraction

Total RNA was obtained from fresh leaves of infected cucurbit crop as described by [17]. The plant tissues were triturated in 2 volumes of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA, 500 mM sodium chloride and 0.1% 2-mercapthoethanol) and centrifuged at low speed in 2 ml eppendorf tube. After addition of 50 µL 20% SDS, the extract was kept at 65ºC for 15 min. Then 250 µL of 6M potassium acetate (pH 6.5) were added and the tubes were transferred on ice for 20 min. After centrifugation, nucleic acids were precipitated with 500 µL ethanol. The pellet was resuspended in 50 µL RNAse free sterile water.

2.4 RT-PCR

RT-PCR reactions were performed in a thermal cycler (Techne TC 4000, Cambridge, UK) using CI specific primers CI-5' (5'-GGCTTCTGAGCAAAGATG-3') and CI-3' (5'-CCCAYCAACTGTYGGAAG-3') [18]. The complementary DNA synthesis was done in a PCR tube containing 2 µL of total RNA, 2 µL reverse primer (10 mM) and 20 µL of RNAse free sterile water. This mix was heated at 70ºC for 2 min and cooled for 90 sec. Ten µL of reverse transcriptase buffer (5X), 2 unit of MMLV-RT (Fermentas), 2 unit of RNAse inhibitor (RNAsin, Fermentas), 0.6 µL of dNTPs (25 mM) and 20 µL of sterile water were added and the mix was incubated at 42ºC for 60 min. PCR was

Fig. 2. Map of study area where samples were collected

performed using 8 µL of *Taq* DNA polymerase buffer (Fermentas), 2 µL of dNTPs (25 mM), 5 units of *Taq* polymerase (Fermentas), 2 µL of each primer, 33.6 µL of sterile water and 2 µL of the cDNA. PCR reactions were performed by initial denaturation of 2 min at 94ºC followed by 35 cycles of 30 sec at 94ºC, 30 sec at 53ºC and 2 min at 72ºC. Final extension was 10 min at 72ºC. Ten µL of PCR product were analysed using electrophoresis through 1% agarose gel.

2.5 Amplicon Purification, Sequencing and Sequence Analysis

The amplicons were purified according to manufacturer's instructions with the Roche High Pure PCR Product Purification Kit. Sequencing was done by using automated DNA sequencer (Applied Biosystems ABI 310) at Inqaba Biotech West Africa (IBWA), Ibadan, Nigeria. The nt sequences were compared with known sequences in the GenBank available at National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTn) program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence identities were calculated from the sequence identity matrix option in MEGA 6 window software [19].

3. RESULTS

3.1 Serology Test

The results obtained from this test showed that out 24 samples tested, 20 samples reacted positively against potyvirus specific antiserum while 4 isolate reacted negatively. The samples that reacted positively included Watermelon virus isolate from Akamkpa, Watermelon virus isolate from Ehom, Telfairia virus isolate from Akpet, Telfairia virus isolate from Abini, *Cucurbita pepo* virus isolate from Adim, *Cucurbita pepo* virus isolate from Iwuru, *Zeneria spp* virus isolate from Nko, *Lagenaria siceraria* virus from Mkpani, *Lagenaria siceraria* virus from Ofatura, *Trichosanthes Cucumerina* from Okuni, *Trichosanthes Cucumerina* from Ikom, *Momordica charantia* from Ugep, *Cucurbita pepo* from Afrike, *Zeneria spp* virus isolate from Alege, *Cucumeropsis mannii* from Igwo, *Cucurbita pepo* from Igoli, *Momordica charantia* from Okuku, Telfairia virus isolate from Sankwala, Watermelon virus isolate from Basang and *Momordica charantia* from Udeshi . Samples that reacted negatively included Cucumber virus isolate from Calabar and Baccocco, *Cucurbita pepo* virus isolate from Ekori and *L. siceraria* virus from Ofodua. Samples were considered virus positive when the optical density (OD) reading at A_{405nm} was twice greater than the absorbance from healthy controls (Table 1).

3.2 Gel Electrophoresis

The result for the amplification of the cDNA for the potyviruses is shown in Fig. 2. The amplified cDNA bands for lane 1 to 24 corresponded to 700 bp on the ladder which is the approximate base pair for potyvirus cylindrical inclusion (CI) amplification.

Lane 1 is Cucumber virus isolate from Calabar, 2 is Cucumber virus isolate from Baccocco, 3 is watermelon virus isolate from Akamkpa, 4 is watermelon virus isolate from Ehom, 5 is Telfairia virus isolate from Akpet, 6 is Telfairia virus isolate from Abini, 7 is *Cucurbita pepo* virus isolate from Adim, 8 is *Cucurbita pepo* virus isolate from Iwuru, 9 is *Cucurbita pepo* virus isolate from Ekori, 10 is *Zeneria spp* virus isolate from Nko, 11 is *Lagenaria siceraria* virus isolate from Mkpani, 12 is *L. siceraria* virus isolate from Ofodua, 13 is *L. siceraria* virus isolate from Ofatura, 14 is *Trichosanthes Cucumerina* virus isolate from Okuni, 15 is *Trichosanthes Cucumerina* virus isolate from Ikom, 16-*Momordica charantia* virus isolate from Ugep, 17 is *Cucurbita pepo* virus isolate from Afrike, 18 is *Zeneria spp* virus isolate from Alege, 19 is *Cucumeropsis mannii* virus isolate from Igwo, 20-*Cucurbita pepo* virus isolate from Igoli, 21 is *Momordica charantia* virus isolate from Okuku, 22 is Telfairia virus isolate from Sankwala, 23-Watermelon virus isolate from Basang, 24 is *Momordica charantia* virus isolate from Udeshi while lane H is for healthy control and D is disease sample.

3.3 Nucleic Acid Sequencing and Sequence Analysis

A fragment of the predicted size, 700 bp, was obtained by RT-PCR. Result of gene sequence analysis showed that the virus isolate from watermelon in Akamkpa, L. *siceraria* isolate from Mkpani and Ofatura have 85 to 99% sequence identity with Morroccan watermelon mosaic virus with Genbank accession of KU315179.1. The result further revealed that *C. mannii* virus isolate from Igwo and watermelon virus isolate from Ehom have 85 and 95% sequence identity respectively with Papaya ringspot virus with

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

S/N	Host/Isolate	Origin	Senatorial District	OD reading at A_{405nm} against virus Polyclonal Antibody for Potyvirus
1.	Cucumber virus isolate	Calabar	South	0.321
2.	Cucumber virus isolate	Baccocco	South	0.700
3.	Watermelon virus isolate	Akamkpa	South	1.749*
4.	Watermelon virus isolate	Ehom	South	1.270*
5.	Telfairia virus isolate	Akpet	South	0.986*
6.	Telfairia virus isolate	Abini	South	1.105*
7.	*C. pepo* virus isolate	Adim	South	0.998*
8.	*C. pepo* virus isolate	Iwuru	South	1.911*
9.	*C. pepo* virus isolate	Ekori	Central	0.698
10.	*Zeneria spp* virus isolate	Nko	Central	1.006*
11.	*L. siceraria* virus isolate	Mkpani	Central	1.847*
12.	*L. siceraria* virus isolate	Ofodua	Central	0.177
13.	*L. siceraria* virus isolate	Ofatura	Central	1.885*
14.	*T. cucumerina* virus isolate	Okuni	Central	0.981*
15.	*T. cucumeria* virus isolate	Ikom	Central	1.891*
16.	*M. charantia* virus isolate	Ugep	Central	0.899*
17.	*C. pepo* virus isolate	Afrike	North	1.221*
18.	*Zeneria spp* virus isolate	Alege	North	0.934*
19.	*C. mannii* virus isolate	Igwo	North	1.001*
20.	*C. pepo* virus isolate	Igoli	North	1.715*
21.	*M. charantia* virus isolate	Okuku	North	1.900*
22.	Telfairia virus isolate	Sankwala	North	1.911*
23.	Watermelon virus isolate	Basang	North	0.912*
24.	*M. charantia* virus isolate	Udeshi	North	1.013*
Healthy Control				0.405
Infected Control				1.894

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

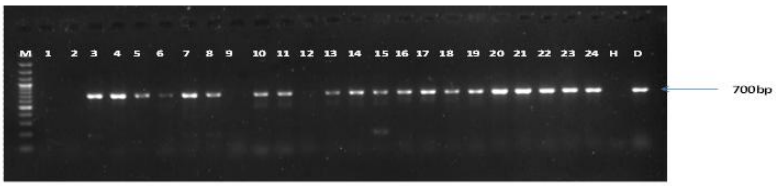

Fig. 3. Amplification of cDNA bands of isolates testing for cylindrical inclusion (CI) of approximately 700 base paris

Genbank accession of KF033100.1. Again, *M. charantia* virus isolate from Okuku and Telfairia virus isolate from Akpet and have 88 and 91% sequence identity respectively with Zucchini tigre mosaic virus with Genbank accession MN267689.1. Telfairia virus isolate from Abini has 90% sequence identity with Potato virus Y with Genbank accession AF321554.1. *C. pepo* virus isolate from Adim and Iwuru both have 86% sequence identity Watermelon mosaic virus with KU352744.1 as Genbank accession. *Zeneria spp* virus isolate from Nko, *T. Cucumerina* from Okuni and Ikom, *M. charantia* virus isolate from Ugep, *C. pepo* virus isolate from Afrike, Telfairia virus isolate from Sankwala, Watermelon virus isolate from Basang and *M. charantia* virus isolate from Udeshi have 80 to 92% sequence identity with Algerian watermelon mosaic virus with Genbank accession KU352744.1. *Zeneria spp* virus isolate from Alege and *C. pepo* virus isolate from Igoli have 81 and 91% sequence identity with Soybean mosaic virus with Genbank accession HQ396719.1 (Table 2).

Table 2. Gene sequence analysis with genbank accession number, Host/isolates,% identity and origin of virus isolates

S/N	Host/Virus isolate	Viruses	Origin	Senatorial District	Accession Number	% Identity
1.	Watermelon	MWMV	Akamkpa	South	KU315179.1	89
2.	Watermelon	PRSV	Ehom	South	KF033100.1	86
3.	Telfairia	ZTMV	Akpet	South	MN267689.1	91
4.	Telfairia	PVY	Abini	South	AF321554.1	90
5.	*C. pepo*	WMV	Adim	South	KU352744.1	86
6.	*C. pepo*	WMV	Iwuru	South	KU352744.1	86
7.	*Zeneria*	AWMV	Nko	Central	KU352744.1	80
8.	*L. siceraria*	MWMV	Mkpani	Central	KU315175.1	85
9.	*L. siceraria*	MWMV	Ofatura	Central	KU315175.1	87
10.	*T. Cucumerina*	AWMV	Okuni	Central	KU352744.1	87
11.	*T. Cucumerina*	AWMV	Ikom	Central	KU352744.1	92
12.	*M. charantia*	AWMV	Ugep	Central	KU352744.1	83
13.	*C. pepo*	AWMV	Afrike	North	KU352744.1	89
14.	*Zeneria spp*	SMV	Alege	North	HQ396719.1	81
15.	*C. mannii*	PRSV	Igwo	North	KF033100.1	95
16.	*C. pepo*	SMV	Igoli	North	HQ396719.1	91
17.	*M. charantia*	ZTMV	Okuku	North	MN267689.1	88
18.	Telfairia	AWMV	Sankwa	North	KU352744.1	81
19.	Watermelon	AWMV	Basang	North	KU352744.1	81
20.	*M. charantia*	AWMV	Udeshi	North	KU352744.1	86

4. DISCUSSION

4.1 Serology Test

In this study, 24 virus infected leaf samples were obtained from cucurbits grown across major cucurbit growing regions in Cross River State, Nigeria. Samples were tested against Antigen coated plate (ACP) enzyme linked immunosorbent assay (ELISA), 20 samples tested positive against the universal potyvirus antisera while 4 samples reacted negative. This result agrees with the work of [12] and [20] who reported potyvirus infecting *Telfairia occidentalis* and cucumber respectively using ACP-ELISA as diagnostic tool. The result further corroborates the report by [21] who employed ACP-ELISA in the detection of plant viruses in *Dioscorea species.*

4.2 RT-PCR and Gene Sequence Analysis

The 20 samples were further detected by RT-PCR with a predicted size of 700 bp using potyvirus cylindrical inclusion (CI) primers. The gene sequence analysis revealed that Algerian watermelon mosaic virus was predominant representing 40% of total viruses detected followed by Morroccan watermelon mosaic virus representing 15% of total viruses detected followed by Papaya ringspot virus, Zucchini tigre mosaic virus, Watermelon mosaic virus and Soybean mosaic virus representing 10% each for total viruses detected and finally Potato virus Y representing 5% of total virus detected. 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. Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times [22,23,24]. This result supports the report of [2,25] and [26] who employed RT-PCR and gene sequence analysis in the detection of plant viruses. Report by [27] and [18] have also validated the use of RT-PCR and gene sequence analysis in detecting plant viruses in the genus *Potyvirus.*

5. CONCLUSION

This study was a survey on occurrence, pathogenicity and identification of viruses infecting some cucurbit crops across major

cucurbit-growing areas in Cross River State, Nigeria and was aimed at identifying viruses infecting cucurbits, ACP-ELISA and RT-PCR were employing as diagnostic tools. According to the results of this study, 20 out of 24 samples tested positive against universal potyvirus antisera, while results obtained from gene sequence analysis revealed that cucurbits grown in Cross River State are severely infected with AWMV, MWMV, PRSV, PVY, WMV, ZTMV and SMV. This survey is an accurate and timely detection and will form the basis for mitigating yield losses resulting from virus infection on cucurbits. This research could be adopted as a template for subsequent related researches.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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