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Identification of Mixed Virus Infection on *Trichosanthes cucumerina* L. in Akamkpa, Southern 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 and DEA managed the analyses of the study. Author ATO read the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Trichosanthes cucumerina L is a tropical or subtropical fruit bearing crop of the Cucurbitaceae family. Field survey during 2020 planting season in Akamkpa, Southern Cross River State, Nigeria revealed widespread virus infection of the crop. Symptoms observed included severe leaf malformation/reduction and rugosity. This research was therefore aimed at identifying viruses infecting *Trichosanthes cucumerina* in this location. Infected leaf samples were collected and virus maintained on young seedlings of *T. cucumerina* in the Botanical Garden of University of Calabar, Nigeria. Diagnostic tools included ACP-ELISA and gene sequence analysis. The ELISA result revealed two viruses belonging to the genus *Cucumovirus* and *Potyvirus*. The identity of the viruses were determined after virus sequences were compared with other viruses available in the GenBank of National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLASTn). The result revealed the *Cucumovirus* to be *Cucumber mosaic virus* (CMV) while the *Potyvirus* was revealed to be *Potato virus Y* (PVY). This is the first report of mixed virus infection on *Trichosanthes cucumerina* in Nigeria.

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1. INTRODUCTION

Trichosanthes cucumerina L is a tropical or subtropical species of the Cucurbitaceae family. Its variety *T. cucumerina* var. *anguina* is raised for its strikingly long fruit. In Asia, it is eaten immature as a vegetable much like the summer squash and in Africa, the reddish pulp of mature snake gourd is used as an economical substitute for tomato. Common names for the cultivated variety include snake gourd [1]. The common name "snake gourd" refers to the narrow, twisted and elongated fruit. The soft-skinned immature fruit can reach up to 150 cm (59 in) in length. It is soft, bland, somewhat mucilaginous flesh is similar to that of the luffa and the calabash. It is popular in the cuisines of South Asia and Southeast Asia and is now grown in some home gardens in Africa. In some cultivars, the immature fruit has an unpleasant odour and a slightly bitter taste, both of which disappear in cooking. The fruit becomes too bitter to eat as it reaches maturity, but it does contain a reddish pulp that is used in Africa as a substitute for tomatoes. The shoots, tendrils and leaves are also eaten as greens [1].

Based on its soil and climate properties, Akamkpa Southern Cross River State, Nigeria has great potential for producing several cultivated cucurbit species, specifically melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum; Nakai), and squash (*Cucurbita* spp.). However, *T. cucumerina is a major grown cucurbit* in this region because it serves as alternative to tomatoes which climatic condition does not support it growth. Several biological factors, mainly insect and disease infection can affect cucurbit production. Viral diseases are of great importance in this respect, because they can affect fruit quality and quantity, reducing production by up to 100% [2].

At least 60 viruses can infect plants in the Cucurbitaceae family, and new virus species on these hosts are described every year [3,4,5]. These viruses have emerged as the most economically significant viruses infecting cucurbits in various cucurbit-growing regions of the globe [6]. Nine of these viruses are seedborne in melon and are transmitted by beetles. Others are transmitted mechanically and by several aphid species in a non persistent manner [7]. The infection incidence with these viruses is high and they can occur as single or mixed infections with other virus species, including *Cucumber mosaic virus* (CMV of the *Cucumovirus* genus) [7].

Mixed virus infections in plants can exacerbate and increase the severity of disease symptoms, significantly decreasing the quantity and quality of crop produced [8]. CMV is a viral species that infects plants, and has the largest known host range. It can also synergistically interact with viruses from the genus *Potyvirus*, increasing disease symptoms and damage to the crop [9]. A visit to some farms in this region revealed wide spread infection of this crop, infected leaves exhibited virus like symptoms of rugosity and severe leave reduction. Many viruses have been characterised and identified in different parts of Nigeria. For example [10] and [11] have reported CMV infecting *Ocimum gratissimum* and *Algerian watermelon mosaic virus* infecting cucumber in Calabar respectively. However, there are no information on the identity of viruses infecting *T. cucumerina* hence this study. Thus, this research was conducted to identity viruses infecting *T. cucumerina* using serological and gene sequence analysis methods.

2. MATERIALS AND METHODS

Leaf samples with virus symptoms were collected from *T. cucumerina* growing in the field of Akamkpa Southern Cross River State, Nigeria during the 2020 farming season. The leaf samples with typical virus symptoms were maintained on young seedlings of *T. cucumerina* in the Botanical Garden of University of Calabar, Calabar and later transported to the molecular laboratory of National Institute of Horticulture (NIHORT) at Ibadan, Nigeria for serological testing, RNA extraction and RT-PCR while the amplicons were thereafter transported to the virology laboratory of International Institute of Tropical Agriculture (IITA) at Ibadan, Nigeria for sequencing.

2.1 Serological Tests

Antigen coated plate enzyme linked immunosorbent assay (ACP-ELISA) as described by [12] was used to detect the genus to which the viruses belongs. The infected leaf sample of 0.1g *T. cucumerina* 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). Cucumovirus and universal potyvirus antisera were diluted at 1:3000 in the adsorption solution and 100 µL of each polyclonal antisera was added to wells 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 microlitres 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 microlitres of 0.001g·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 [13]. Healthy plant sample 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 [12].

2.1.1 RNA extraction from infected leaf samples

Total RNA was obtained from fresh leaves of infected *T. cucumerina* plant as described by [14]. Plant tissue was 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 Eppendorff 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.

2.1.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR reactions were performed in a thermal cycler (Techne TC 4000, Cambridge, UK) using cylindrical inclusion (CI) primers forward 5'-GGIVVIGTIGGIWSIAARTCIAC-3', Reverse 3' ACICCRTTYTCDATDATRTTIGTIGC-5' and CMV primers Forward 5'-TGGTCGTCCAACTATTAACCAC-3' Reverse 3'-TACTGATAAACCAGTACCGGTGA-5' as described by [11]. 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 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.

2.1.3 Amplicon purification and sequencing

The RT-PCR amplicons were purified by adding 95 % ethanol to 40 µl of the amplicons 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. 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 cDNA was dissolved in 30 µl of sterile distilled water. The product was sequenced at the Bioscience Laboratory of the International Institute of Tropical Agriculture (IITA) at Ibadan, Nigeria.

2.2 Sequence Analysis

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

3. RESULTS AND DISCUSSION

3.1 ACP-ELISA Result for Pot Virus and CMV Detection

Field observation in Akamkpa southern Cross River State, Nigeria during 2020 planting season revealed widespread virus infection of *Trichosanthes cucumerina*. The results obtained from this test showed that the virus isolate reacted positively against the CMV and universal potyvirus antisera (Table 1). The optical density reading for the CMV was 0.894 while the result obtained for the potyvirus was 1.007 which are twice greater than 0.326 and 0.405 respectively for the optical density reading for healthy control.

3.2 Nucleic Acid Sequencing and Sequence Analysis

A fragment of the predicted size of 700 bp was obtained by RT-PCR for the potyvirus cylindrical inclusion (CI) primers and 500 bp for the CMV primers. The gene alignment of the potyvirus and CMV sequences with other viruses available in the GenBank revealed 90 % sequence identity with *Potato virus Y* (PVY) with accession number of JF707767.1 and 93 % sequence identity with Cucumber mosaic virus isolate HM3 Segment RNA1 with accession number of KT921314.1 9 respectively.

In this study, *Potato virus Y* (PVY) as a *Potyvirus* and *Cucumber mosaic virus* (CMV) as a *Cucumovirus* were detected from a naturally infected *Trichosanthes cucumerina*. The plant exhibited severe leaf reduction and rugosity symptoms when characterised serologically and molecularly.

The ACP-ELISA test result revealed a mix infection of potyvirus and CMV. [15,4,16,5,17,18] have reported the detection and identification of plant viruses into the genus taxon using ACP-ELISA.

Fig. 1. Severe leaf reduction and rugosity on *Trichosanthes cucumerina*

Table 1. 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	
		CMV	Potyvirus
Trichosanthes cucumerina	Akamkpa	0.894*	1.007*
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_{405nm} was 2x greater than the absorbance from healthy controls*

The result obtained from gene sequence analysis revealed the potyvirus and CMV in this study to be *Potato virus Y* (PVY) and *Cucumber mosaic virus*. Gene sequencing as tool for virus identification and characterization has become the ultimate in recent times [5,19,20,21,22, 8,23,24]. Eyong et al., (2020) have reported the identification of CMV and *Algerian watermelon mosaic* virus through molecular characterisation. Desbiez et al., (2012) have also employed molecular tools in the detection of plant viruses. [25,1] and [13] have reported mix virus infection on some cucurbit hosts.

4. CONCLUSIONS

According to the results of this study, there is mixed virus infection of *Potato virus Y* and *Cucumber mosaic virus* on *Trichosanthes cucumerina* in Akamkpa, Southern Cross River State, Nigeria. The viruses induced serious symptoms on leaves of this species, reducing growth, fruit size and, consequently, fruit quality and quantity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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