A-636
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RESEARCH ARTICLE Indian Journal of Agricultural Research,

Detection of a New Strain of the *Yambean mosaic virus* in *Senna hirsuta* (Irwin and Barneby) in Calabar, Southern Nigeria

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ABSTRACT

Background: Hairy senna is an edible weed grown for both its vegetables and as a hedge plant in farms and backyard gardens in Calabar. *Senna hirsuta* leaf samples showing virus-like symptoms were collected in the growing seasons of between 2018 and 2020 from backyard gardens and farms in Calabar and the surrounding area.

Methods: The virus was isolated and transmitted by mechanical inoculation for host range studies. Seed transmissibility and transmission by aphids were further used for detection. Reverse transcription polymerase chain reaction (RT-PCR) using primers specific to the cylindrical inclusion protein gene (CI) of potyviruses followed by sequencing was used for molecular identification. The neighbour-joining phylogenetic tree was constructed to determine the relationship of the Calabar isolate with those from other parts of the world.

Result: For the host range studies, four species belonging to the Fabaceae family were infected by the isolate, indicating a narrow host range. The virus was found to be seed transmissible and could be vectored by both *Aphis craccivora* and *Aphis spiraecola*. Obtained DNA fragments with the expected size of~700 bp were amplified and a pairwise comparison with other potyviruses revealed a 94-95.87% similarity with complete sequence of YBMV (JN190431). Phylogenetic analysis placed the senna isolate in the same clade as the Brazilian isolates (MK825543 and MK825544) and JN190431. On the basis of this, the virus is adjudged to be the Nigerian strain of YBMV (JN190431).

Key words: ACP-ELISA, Cylindrical inclusion, Molecular detection, Potyvirus, *Senna hirsuta,* YBMV, YBMV-Ng.

INTRODUCTION

Potyviridae is the largest family of plant-infected RNA viruses usually resulting in serious disease epidemics in a wide range of cultivated plants (Revers and Garcia 2015; Wylie *et al*. 2017). In terms of genomic organization, members of the family Potyviridae consist of monopartite and bipartite plant viruses with single-stranded and positive-sense RNA genomes, ranging in size from 8.2 kb to 11.3 kb with an average size of 9.7 kb. The flexuous filamentous particles with no envelope are 680-900 nm long and 11-20 nm in diameter encompassed by a single core capsid protein (Wylie *et al*. 2017).

The family Potyviridae is made up of 12 definitive genera namely Arepavirus, Bevemovirus, Brambyvirus, Bymovirus, Celavirus, Ipomovirus, Macluravirus, Poacevirus, Potyvirus, Roymovirus, Rymovirus and Tritimovirus and they are distinguished by host range, genomic features and phylogeny (Wylie *et al*. 2017; Walker *et al*. 2020). Most potyviruses have been reported to infect dicotyledons, but only a few can infect monocotyledons (Revers and Garcia 2015; Wylie *et al*. 2017; Lefkowitz *et al*. 2018).

Senna hirsuta (H.S. Irwin and Barneby) is a tropical herbaceous weed belonging to the Fabaceae family. It is found on roadsides, wastelands and along forest boundaries of some tropical and subtropical regions of the world. Essiett and Bassey (2013) and Sofowora (2008) reported the use of *S. hirsuta* leaves in the treatment of herpes and for laxative and purgative purposes. The use of its seeds in Laos as a substitute for coffee and leaf decoction against skin irritation in Thailand has also been reported (Hanum and Maesen, 1997) and its oil is used for antimicrobial purposes (Essien *et al*. 2019).

Several weeds have been reported to serve both as natural hosts and reservoirs for plant viruses such as African cassava mosaic virus (ACMV) in *Centrosema pubescens* (Ekpiken and Eyong 2022), Beet western yellow virus (BWYV) and Pepper mild mottle virus (PMMoV) in *Leonurus sibricus* (Kwon *et al*. 2016), Cucumber mosaic virus (CMV)

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How to cite this article: Ekpiken, E.E., Eyong, O.I., Adediji, A.O., Mofunanya, A.A.J., Arogundade, O. and Owolabi, A.T. (2023). Detection of a New Strain of the *Yambean mosaic virus* in *Senna hirsuta* (Irwin and Barneby) in Calabar, Southern Nigeria. Indian Journal of Agricultural Research. doi: 10.18805/IJARe.A-636.

Submitted: 26-03-2021 **Accepted:** 03-02-2023 **Online:** 25-02-2023

in *Eleutheranthera ruderalis* (Arogundade *et al*. 2019) and Cowpea aphid-borne mosaic virus (CABMV) in *Senna occidentalis* (Kitajima *et al*. 2008).

Potyviruses have been described as constituting the largest group of known plant viruses and they are responsible for significant losses in crop yields (Wylie *et al*. 2017).

The *S. hirsuta* weed is particularly interesting because it is used as a hedge plant in most of the farms visited. Subsistence and small-scale farmers practice mixed cropping for the purpose of maximizing land used for the cultivation of crops and at such farms it was a common sight to see leaves exhibiting symptoms of viral infection. *Sphenostylis stenocarpa* L. an underutilized leguminous crop found in some of the farms visited was particularly of interest as it exhibited the same foliar symptoms as witnessed on *S. hirsuta.*

MATERIALS AND METHODS

Disease incidence

In the three seasons of the survey (2018, 2019 and 2020), a total of 114 young symptomatic leaf samples from *S. hirsuta* were collected from gardens and small farms located in the Anantigha, Baccoco, Atimbo and Lemna areas of Calabar. The bags were then placed in Ziplock polyethylene bags, labeled and placed on ice to be taken to the laboratory for determination of the incidence of the disease. The incidence of the disease was evaluated by adopting Folarin *et al*. (2020) and the incidence was scored as the presence or absence of symptoms of the virus disease using a rating scale where low incidence = 1%-20%; moderate incidence = 21%-49%; high incidence = 50%-100%. (Were et al. 2004).

The percentage of disease incidence was calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{X_0}{X_1} \times 100$$

X_0 = Number of plants suspected to be infected.
X_1 = Total number of plants.

All leaf samples collected from the field were subjected to antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) as described by Kumar (2009), for the presence of the virus using antibodies specific for *Cucumber mosaic virus* (CMV), *Bean common mosaic virus* BCMV), *Blackeye cowpea mosaic virus* (BICMV), *Cowpea aphid-borne mosaic virus* (CABMV) and *Yambean mosaic virus* (YBMV).

Virus isolation, host range and symptomatology

Symptomatic leaves obtained from *S. hirsuta* were collected from both farms and backyard gardens in Calabar, Cross River State, Nigeria, in sealed polyethylene bags between the 2018 and 2020 growing seasons and placed on ice to ensure the viability of the virus. Infected leaf tissues were crushed in 0.03M sodium phosphate buffer pH 8.0 in a precooled oven sterilized pestle and mortar (Fig 1).

For the host range study, the inoculum obtained from infected leaves was inoculated on a variety of test plants by rubbing 600 mesh carborundum dusted on leaves of 15 plant species spread across four families. The inoculated leaves were washed with water immediately after inoculation to remove the residual carbohydrate. Inoculated plant species were kept in the greenhouse of the University of Cross River State, Calabar and symptoms were regularly recorded and infection status was confirmed by an enzyme-linked immunosorbent assay (ACP-ELISA) on apical leaves, 4-6 weeks after inoculation.

Aphid transmission test

Virus-free colonies of *Aphis craccivora* Koch*, A. spiraecola* Patch and *Aphis citricidus* Kirkaldy were collected from the laboratory and maintained in screen cages on *Phaseolus vulgaris* L., *Cucumis sativus* L. and seedlings of *Citrus aurantifolia* (Christm.) Swingle, respectively, before being used for transmission test. Cowpea seedlings (*Vigna unguiculata* L.) grown from certified virus-free seeds were used as test plants for the studies. To maintain the same virus concentration, virus source plants were used one month after inoculation. The aphids were starved for an hour and allowed to feed on symptomatic leaves of *S. hirsuta* for 5 minutes. Five aphids were transferred to *Vigna unguiculata seedlings* and allowed an inoculation access period. Post-inoculated plants were sprayed with Lambda (Cyhalotrin) and kept in insect-proof cages to record symptoms. The test plants were observed for 21 days and the expression of the symptoms was checked with further confirmation by ACP-ELISA.

Seed transmission test

To evaluate seed transmission, seed samples obtained from pods of previously inoculated seedlings of *S. stenocarpa* and *S. hirsuta* were harvested. One hundred (100) mature seeds from *S. stenocarpa* and *S. hirsuta* were individually grown in trays (30.5 cm × 16.5 cm) containing steam-sterilized soil in an insect-proof screenhouse to determine the percentage of seed transmission. Seed-borne infection was evaluated for the presence of the virus on young leaves by subjecting them to the ACP-ELISA test.

Serological test

A universal potyvirus and CMV-specific antisera were used in an Antigen Coated Plate Enzyme-linked Immunosorbent assay (ACP-ELISA) as described by Kumar (2009) to determine the genera to which the virus isolate belonged. A polyclonal antiserum specific for Cucumber mosaic virus (CMV) and potyvirus genus-specific antibodies (AS-573/1; DSMZ) were obtained from Agdia incorporated, USA and the German Resource Center for Biological Material, Braunschweig, Germany, to detect the virus from leaf extracts in PBS-Tween buffer. The absorbance was read at 405 nm using a microtitre plate reader. A sample was considered positive when its absorbance was at least twice that of the healthy control (Sutula *et al.,* 1986).

RNA extraction, electrophoresis and sequence analysis

Total RNA was isolated from 0.1 g of leaf samples of symptomatic and asymptomatic *S. hirsuta* plants using a

cetyltrimethylammonium bromide (CTAB) protocol described by Abarshi *et al.* (2010). Extracted RNA was converted into a complementary DNA (cDNA) through RT-PCR as described by Pappu *et al*. (1993) then cDNA was amplified with a degenerate primer pair for potyvirus CIF/CIR (5′ GGIVVIGTIGGIWSIGGIAARTCIAC 3′/5′ACICCRTTYTC DATDATRTTIGTIGC 3′) (Ha *et al*. 2008).

The amplification was carried out on a GeneAmp 9700 PCR system thermocycler (Applied Biosystem Incorporated, USA) using the following thermocyclic conditions: 42°C for 30 minutes for reverse transcription, 94°C for 3 minutes for initial denaturation, followed by 40 cycles of denaturation at 94°C for 30 seconds, an annealing step at 40°C for 30 seconds, an extension at 68°C for 1 minute and a final extension at 72°C for 10 minutes ending 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. The amplicon was purified by adding 2.5 volumes of 95% ethanol to 40 µl of the amplicon in a new 1500 µl Eppendorf tube and the solution kept at -80°C for 10 minutes. The tube was centrifuged at 12000 rpm for 10 minutes and the supernatant was discarded. 500 µl of 70% ethanol was again added to it and centrifuged at speed of 12000 rpm for 5 minutes. The supernatant was discarded and the tube was left at room temperature to dry the purified cDNA after which the purified product was dissolved in 30 µl of sterile distilled water. The purified preparation was obtained and then sequenced.

Sequence and phylogenetic analysis

The sequence obtained was compared with known viral sequences using the Basic Local Alignment Search Tool (BLASTn) program available at the National Center for Biotechnology Information (NCBI) for species identification and sequence homology. Multiple and pairwise alignments were achieved using CLUSTALW and BioEdit version 7.2.5 (Hall 2013) Pairwise sequence comparisons were computed using Sequence Demarcation Tool (SDT) version 1.2 (Muhire *et al.* 2014) with the MUSCLE algorithm (Edgar 2004) used as the alignment option. Phylogenetic reconstruction was performed by MEGA version 6 (Tamura *et al.* 2013) using the Neighbor-joining method (Maximum composite model) with selected viruses of the same genus. Bootstrap values were calculated using 1000 random replications.

RESULTS AND DISCUSSION

Incidence of YBMV

A total of thirty-two fields were visited in the three seasons, with the collected leaf samples showing typical mosaic, leaf distortion, green vein banding and stunting of *S. hirsuta*. Across the four districts within Calabar, the highest mean incidence of disease (Table 1) was observed in the Baccoco district (46.1%, 40.0% and 33.3%) for the growing seasons of 2018, 2019 and 2020, respectively, while the lowest incidence of 16.6%, 20% and 25 was observed in the Lemna district. Disease incidence recorded in Anantigha was 42.8%,

Fig 1: (a) Leaf malformation and mosaic on *Senna hirsuta*, (b) Mosaic on *Sphenostylis stenocarpa*, (c) vein clearing on *Phaseolus vulgaris*.

Table 1: Disease incidence of YBMV on *S. hirsuta* in Calabar environs.

District	Season	Number of fields	Number of samples	Mean incidence rate (%)
Anantigha	2018	1	7	42.8
	2019	3	10	30.0
	2020	4	12	33.3
Baccoco	2018	4	13	46.1
	2019	2	10	40.0
	2020	3	15	33.3
Atimbo	2018	2	6	16.6
	2019	2	8	37.5
	2020	3	10	30.0
Lemna	2018	2	6	16.6
	2019	3	10	20.0
	2020	3	8	25.0

30% and 33.3% respectively while the incidence for Atimbo was 16.6%, 37.5% and 30% respectively.

Virus isolation, host range and symptomatology

The isolate was found to be sap-transmissible, having a narrow host range that infected only members of the Fabaceae family and inducing symptoms such as mosaic, vein clearing, mottling and leaf malformation in infected plants (Fig 1 and Table 2).

Table 2: Reaction of different hosts inoculated mechanically with the virus isolate from *Senna hirsute.*

Family	Test plant	Symptoms	ELISA reactivity
Solanaceae	*Physalis angulata*	-	-
	Capsicum annuum	-	-
	Solanum lycopersicum	-	-
	Datura stramonium	-	-
	Nicotiana tabacum	-	-
Cucurbitaceae	*Cucumis sativa*	-	-
	Lagenaria breviflora	-	-
	Cucumeropsis mannii	-	-
Tiliaceae	*Corchorus olitorius*	-	-
Fabaceae	*Senna hirsuta*	M, LM	+
	Sphenostylis stenocarpa	M, LM	+
	Canavalia ensiformis	M, LM	+
	Glycine max	-	-
	Cajanus cajan	-	-
	Senna occidentalis	M	+

VC: Vein clearing, LC: Leaf curling, M: Mosaic, LM: Leaf malformation.

Table 3: Transmission of virus isolate by *A. spiraecola, A. craccivora* and *T. citricida*.

Virus isolate	Vector	% Transmission	Mean YBMV ELISA reactivity (A_{405} nm)
S. hirsuta	*A. citricidus*	0 (0/15)	- (0.563)
	A. spiraecola	86 (13/15)	+ (1.607)
	A. craccivora	73.3 (11/15)	+ (1.024)
Healthy			0.442
Infected			1.894

The values of the virus isolates were considered virus positive when the optical density (OD) was reading at A405nm was 2× greater than the absorbance from healthy controls.

Aphid transmission

The isolate under investigation was transmitted in a non-persistent manner by both *A. spiraecola* and *A. craccivora* (Table 3). *Aphis spiraecola, A. craccivora* and *Myzus persicae* have been reported to transmit Senna mosaic virus (SeMV) in a non-persistent manner (Owolabi and Proll 2001). Other aphids that have been reported to transmit potyviruses in a nonpersistent manner include *potato virus Y* by *A. gossypii* (Talukdar *et al*., 2017), *Tomato mild tomato mottle virus* by *Myzus persicae* (Walkey *et al*., 1994), *sunflower mosaic potyvirus* by *A. craccivora* (Singh *et al*., 2005) and *Moroccan watermelon mosaic virus* by *A. spiraecola* (Owolabi and Ekpiken 2014).

Seed transmission

Results from the seed transmission test revealed that out of the 100 seedlings raised from seeds of infected *S. hirsuta* and *S. stenocarpa*, percentage germination was 93 and 87 respectively while 85 and 72 of the germinated respectively showed typical symptoms of viral infection representing 91% and 83% infection rate from the seeds (Table 4). However, a previously reported potyvirus which was tentatively named Senna mosaic virus (SeMV) was not seed transmissible (Owolabi and Proll, 2001) and points to a different virus from

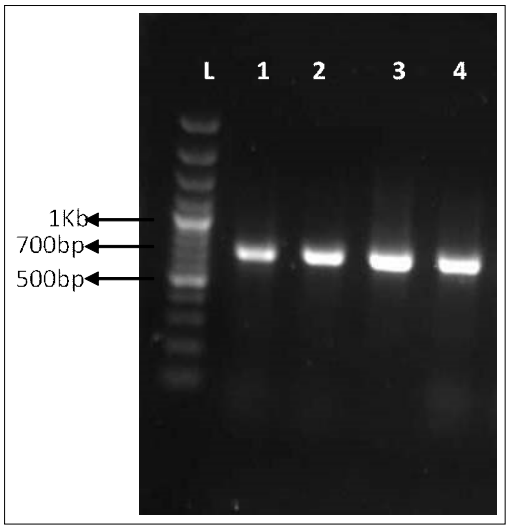

Fig 2: Gel analyses of PCR products targeting potyviruses in *Senna hirsuta* (690 bp). L, 100bp ladder, Lanes 1-4 = samples from symptomatic *Senna hirsuta.* Amplification obtained from primer targeting CI gene of potyvirus gave an expected size of 700 bp.

Table 4: Detection and rate of virus transmission in seeds of *S. stenocarpa* and *S. hirsute.*

Plant	Number of seeds planted	Number of seeds germinated	No. of seeds tested	No. of seeds infected	Rate of seed-borne infection (%)	Mean ELISA reactivity (A_{405} nm)
S. stenocarpa	100	87	87	72	83	+ (1.838)
S. hirsuta	100	93	93	85	91	+ (1.947)
Healthy						0.492
Infected						2.316

Absorbance value (A_{405nm}) is greater than twice of negative control regarded as virus positive.

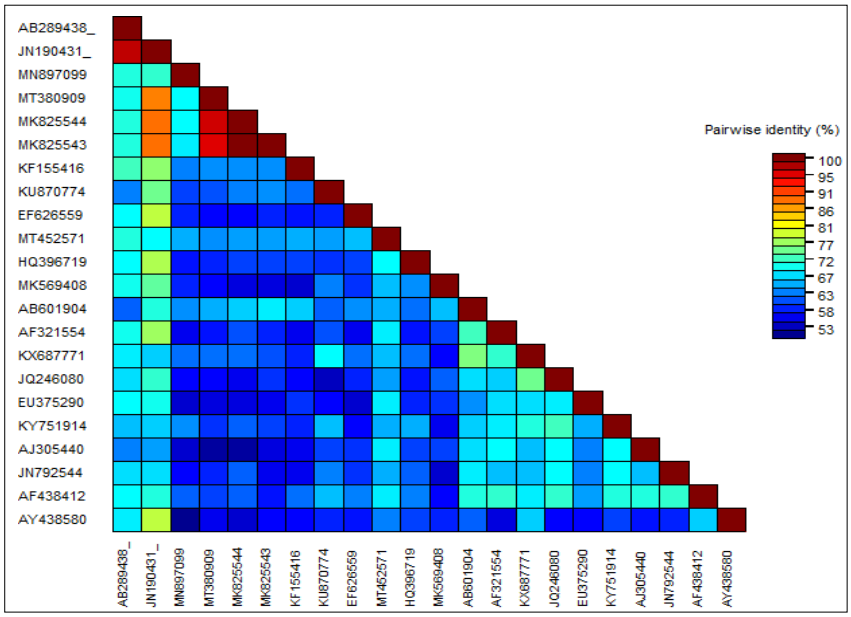

Fig 3: Pairwise sequence comparisons of representative sequences from *Yambean mosaic virus* isolates.

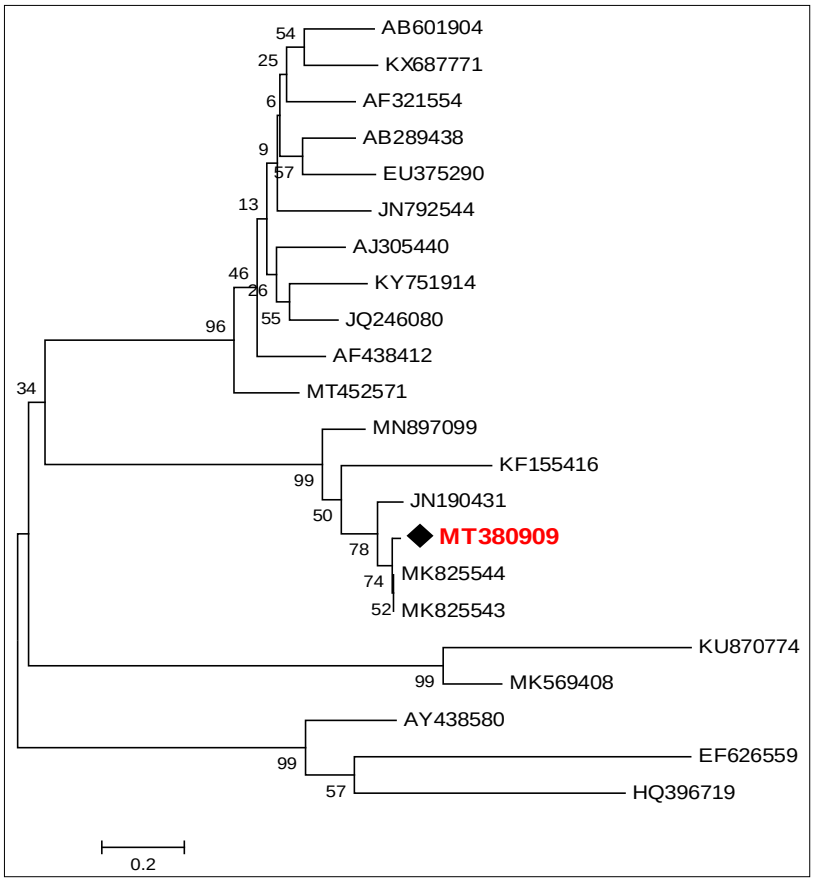

Fig 4: A phylogenetic tree illustrating the relationships between the Nigerian strain of YBMV (MT380909) and other previously reported potyviruses. The phylogenetic tree was generated by the neighbor joining method with 1000 bootstrap replicates using MEGA 6 software. The numbers at the nodes indicate the percentage of 1000 bootstraps occurred in this group. The Nigerian strain of YBMV is highlighted in red.

the one under investigation. Seed transmission has been reported for some members of the genus, *Potyvirus* (Laney *et al*. 2012; Simmons *et al*. 2013; Hajimorad *et al*., 2018). Shukla *et al*. (1994) reported that some potyviruses are seed transmissible, while over 200 species are capable of being transmitted by aphids.

The senna isolate reacted positively against the YBMV antisera. The optical density reading for the virus on *S. stenocarpa* was 1.838 while the optical density value for *S. hirsuta* was 1.947. the healthy control was 0.492 (Table 4). There was no reaction against the CMV antisera.

Sequence analysis and phylogeny

The results of the PCR and gel electrophoresis showed that the nucleic acid of the virus under investigation was amplified using the cylindrical inclusion primer. The PCR products separated on 1.5% agarose gel under the UV transilluminator produced the expected amplicon of 700 bp in size from all symptomatic samples (Fig 2). The 690-bp partial nucleotide sequence obtained was deposited with NCBI GenBank and assigned accession number MT380909. The partial sequence of YBMV shared the highest nucleotide sequence identity of 95.87% with Brazilian isolate 12 of YBMV (MK825544) and aa sequence identity of 94.3% of YBMV (QEQ92495). Pairwise comparisons of the sequence generated from this study with selected sequences of other potyviruses showed that it had a sequence homology of 94-95.87% with YBMV (JN190431) (Fig 3).

A phylogenetic tree was drawn to analyze the phylogenetic relationship of the partial sequence of the virus under study with several selected potyvirus sequences retrieved from the GenBank including a complete genome sequence of YBMV (JN190431). The tree showed that the virus under study was grouped in the same clade with isolates 6 and 12 of YBMV from Brazil and were strains of JN190431 (Fig 4).

Molecular analysis has greatly contributed to determining whether plant viruses can be classified as distinct species or strains of the same species. The development of nucleic acid-based techniques in virus detection with cDNA hybridization and PCR have been used extensively (Makkouk and Kumari, 2006). The criterion for classifying viruses into different species and strains has been defined (Shukla and Ward, 1989; Adams *et al*., 2005; King *et al*., 2011). BLASTn result showed that the virus in this study shared 95.87% sequence homology with Yambean mosaic virus (YBMV) Brazilian isolate 12. Earlier studies on the virus that infected *Pachyrhizus erosus* L. Urb. (Mexican yam bean) was thought to be a strain of BCMV but further studies later established it to be YBMV (de Sá Andrade Medeiros *et al*., 2019; Fuentes *et al*., 2012; Ha *et al*. 2008; Damayanti *et al*. 2008).

CONCLUSION

Weeds are significant in contributing to both the outbreaks of viral diseases in crops and the survival and evolution of viruses in nature (Kaliciak and Syller 2009; Zhao *et al*. 2019). In Calabar, *S. hirsuta* is found in most small holding farms and, being a perennial plant, it could serve as a ready reservoir for YBMV and play an important role in its epidemiology. In nature, potyviruses are transmitted by aphids in a non-persistent manner, which is one of the reasons why they cause serious disease epidemics in a wide range of cultivated plants (Ng and Perry 2004).

The virus under study was found on *S. hirsuta* and was successfully transmitted by seeds and aphids in a nonpersistent manner by *A. spiraecola* and *A. craccivora.* Host range studies in selected host plants showed that it had a narrow host range and was restricted to members of the Fabaceae family. The virus reacted positively against potyvirus-specific antiserum in ACP-ELISA and molecular studies revealed that like the isolates from Brazil (MK825543 and MK825544), the Nigerian isolate was a strain of YBMV (JN190431). This study provides information on the incidence of a potyvirus that infects *S. hirsuta* and establishes that the incidence is moderate. It also establishes that the underutilized legume, *S. stenocarpa* currently receiving attention from research agencies is readily infected by the virus. This provides knowledge necessary for plant breeders to use in their quest for crop improvement. The YBMV isolate from Calabar may also possess unique molecular and evolutionary pattern differentiating it from others which can only be determined using a full genome analysis.

ACKNOWLEDGEMENT

The authors are grateful to the Pan African University for Life and Earth Science Institute (PAULESI), Ibadan, Nigeria for their support.

Conflict of interest: None.

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