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Report on the Detection of a Poty Virus Associated with Mosaic, Leaf Deformation and Stunting on *Ocimum gratissimum* (L.) in Calabar, Nigeria

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Abstract. During a survey of household gardens in Calabar (Nigeria) between the growing seasons 2021 and 2022, yellowing, mosaic and vein banding in the leaves, as well as severe stunting, were reported in scent leaf plants (*Ocimum gratissimum* family Lamiaceae). Diagnostic investigations of host range in the obtained symptomatic samples of the virus suggested a restricted host range, while ACP-ELISA results demonstrated that the isolate reacted positively with the poty virus antisera. Poty virus was further established when a pair of poty virus specific primers produced an amplicon of 700 bp from symptomatic samples. The amplicon was cloned and sequenced before the analysis of the basic local alignment search tool revealed that the cylindrical inclusion (CI) gene showed a 71.11% relationship to the Yam mosaic virus (MG711313). Phylogenetic analysis of the sequence with selected poty virus sequences from NCBI revealed it was nearest in closeness to Yam mosaic virus (MG711313) but was also in the same subgroup with Mullein poty virus (KU962959), Barleria repens mottle virus (ON854904) and PRSV (KF155416). The isolate in this investigation was tentatively determined to be a potyvirus species based on sequence identity at both the nucleic acid and protein levels, as well as phylogenetic analysis.

Keywords: uncharacterized poty virus, ACP-ELISA, RT-PCR, *Ocimum gratissimum,* CI gene

Introduction

The aromatic herb *Ocimum gratissimum* (L.) belongs to the Lamiaceae family. It is popularly known as "African basil," but it is also known locally as "scent leaf," owing to its peculiar aroma. It is a perennial plant that grows throughout the world's tropics and warm temperate zones.

According to Ijeh *et al*. (2004) that the plant is well-known for being used for both medicinal and nutritional purposes. In many homes and restaurants, it is consumed as a leafy vegetable, cooked as a sauce for eating roasted/ boiled plantain or yam. The nutritional value of this crop comes from its ability to be used as a flavouring component in a variety of dishes. *O. gratissimum* is also grown for its essential oils, eugenol and thymol, which may be found in both the leaves and the stems. According to Ijeh *et al*. (2004), eugenol and thymol derived from the oil are suitable alternatives for clove oil and thyme oil. The phytochemical analysis revealed that it was high in flavonoids, alkaloids, phytates, tannins and oligosaccharides (Talabi and Makanjuola, 2017).

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Diseases caused by plant viruses have been reported to cause both qualitative and economic losses in the production of leafy vegetables around the world (Mumford *et al*., 2016). The cultivation of *O. gratissimum* has been plagued by several viruses which include begomo viruses such as *Ocimum* mosaic virus (OcMV), *Ocimum* golden mosaic virus (OcGMV) and *Ocimum* yellow vein virus (OcYVV) as reported by Mollel *et al*. (2020). Other viruses that infect the crop include the tobamovirus, *Ocimum* yellow mosaic virus (Atiri 1999) and *Cucumber* mosaic virus (Ekpiken *et al*., 2021a; Sinha and Samad, 2019; Ayo-John and Hughes, 2014). A mixed infection of a poty virus with CMV has also been reported on *O. gratissimum* (Ekpiken *et al*., 2021b), however there has been no further literature reporting their occurrence on the crop. As at time this research was conducted, there has been no report on the infection of *O. gratissimum* by poty viruses.

Poty viruses belong to the family, *Potyviridae* which is the largest RNA virus family and they are placed within the phylum *Pisuriviricota* (Koonin *et al*., 2020). There are more than 200 known plant virus species in the Potyviridae family at present assigned to twelve genera. These genera include Roymo virus, Bevemo virus, Cela virus, Ipomo virus, Maclura virus, Poace

virus, Bramby virus, Bymo virus, Arepa virus, Rymo virus, Tritimo virus and Poty virus (Pasin *et al*., 2022; Gibbs *et al*., 2020).

They are made up of flexible filamentous particles and have a single stranded positive sense RNA genome that has a size of 8-11 Kb. Some poty viruses can be transmitted by seeds, however, transmission by aphid vectors and mechanical inoculation have been found to be efficient (Gadhave *et al*., 2020; Simmons and Munkvold, 2014).

Symptoms associated with the virus disease in the field infecting *O. gratissimum* include chlorosis, mosaic pattern on leaves, leaf deformation and in extreme cases stunted growth. So far, there has been no report of a poty virus infecting *O. gratissimum* in the Calabar area and indeed Nigeria. The current research was conducted to identify the causal agent eliciting the disease on *O. gratissimum* plant as well as determine the genetic diversity of the virus through its evolutionary tree using tools such as host range studies, aphid transmission studies, serology and molecular diagnostics.

Materials and Methods

Sample collection virus isolation and maintenance. Symptomatic leaves of *O. gratissimum* were collected from five gardens in the Calabar area throughout the growth season of 2021-2022, placed in sealed polyethylene bags and kept at 40 °C until they were removed for further processing. Later, the affected leaves were triturated in a pre-cooled, oven-sterilized mortar with cold inoculation buffer of sodium phosphate buffer (pH 8.0, 0.03M). After healthy *O. gratissimum* seedlings have been given a 600-mesh carborundum dusting and placed in the screenhouse with a temperature range of 28-2 °C, the resulting homogenate was utilized to inoculate the seedlings. Subsequently, the virus was kept in check in early *O. gratissimum* seedlings by routine inoculation.

Host range studies. To test for infectivity, 25 different plant species from various families were chosen. Some of these plants included *Nicotania tabacum, Corchorus olitorus, Datura stramonium, Ageratum conyzoides, Ocimum gratissimum* and *Vigna unguiculata*.

Each of these plant species had ten seedlings that were mechanically inoculated. The plants were then rinsed with water before the status of infection of the plant species was established by using ACP-ELISA to test the apical leaves of inoculated plants, usually 4-6 weeks after inoculation.

Aphid transmission. *Aphis spiraecola* (Patch) and *Aphis craccivora* (C. L. Koch), two aphid species that had been tested and found to be virus-free, were obtained from the institute's entomology laboratory and maintained in screen cages on *Phaseolus vulgaris* L. and *Cucumis sativus* L., respectively, before being used for the transmission test. The transmission process followed the guidelines outlined by Lecoq *et al*. (2001). The aphids were starved for two hours, then allowed to feed for two minutes on symptomatic leaves before being transferred to five healthy seedlings each of *Nicotiana tabacum* and *O. gratissimum*, which were used as test plants, in batches of three for inoculative access feeding, which was allowed for 20 min. The plants post-inoculation, were treated with Lambda (Cyhalotrin) and placed in insect-proof cages. They were then monitored for between 4-6 weeks for symptom expression and verification for disease infection provided by ACP-ELISA.

Serology. The antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) as described by Kumar (2009) was used for serological studies to identify the virus in the ocimum leaf sample. A pair of universal poty virus (Agdia, Elkhart, Indiana) and CMV specific antisera (DMSZ, Germany) were employed for identification. In each well of the ELISA plate, 0.1 g of an *O. gratissimum* infected leaf sample was dispensed after being pulverized in 1 mL of a coating buffer (0.015M $Na_2 Co_3$ + 0.0349M NaH Co_3 + dH_2O). PBS-Tween was used to wash the ELISA plate three times with three minutes between each wash after being incubated at 37 °C for 1 h. 20 mL of conjugate buffer and 1 g of healthy plant material was later pulverised. A ratio of 1:3000 was used for the dilution of universal poty virus in the absorption solution from which 100 L of antisera was introduced into the wells of the ELISA plates before incubation for 1 h at 37 °C. PBS-Tween were afterwards used to wash the ELISA plates three times. An alkaline phosphatase conjugate of 100 µL was diluted in conjugate buffer on a ratio of 1:15000 and incubated at 37 °C for 1 h. The plates were rinsed three times more with PBS-T. Each well received 100 L of 0.001 g/L *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) and were incubated at room temperature for 1 h. ELISA cover

plates were used for all incubations. Healthy plant samples were used as standard control. Absorbance was measured at $A405_{nm}$ with an ELISA plate reader (Micro Read 1000 ELISA Plate Analyzer, USA) after 1 h of incubation. When the optical density value obtained is double that of the equivalent healthy controls, the sample is termed positive for a virus.

RNA extraction, RT-PCR and sequencing. Cetyltrimethylammonium bromide (CTAB) procedure as described by Abarshi *et al*. (2010) was used for extracting total RNA from around 0.1 g of leaf samples of symptomatic *O. gratissimum* plants. The obtained cDNA was then amplified by RT-PCR (Pappu *et al*., 1993). Amplification of cDNA was achieved using a cylindrical inclusion gene primer set having the following sequence: CIF 5'-GGIVVIGTIGGIWSIGGIAARTCIAC-3' and CIR5'ACICCRTTYTCDATDATRTTIGTIGC-3' (Ha *et al*., 2008) were utilized to confirm the presence of poty virus in *O. gratissimum* samples. A GeneAmp 9700 PCR system thermocycler (Applied Biosystems Incorporated, USA) were used for amplification with the thermocyclic setting: 42 °C for 30 min for reverse transcription, 94 °C for 3 min for initial denaturing, 40 cycles of denaturing at 94 °C for 30 sec, an annealing step at 40 °C for 30 sec, an extension at 68 °C for 1 min and a final extension at 72 °C for 10 min to complete the reaction. The products of the reaction from the PCR were separated on 1.5% agarose gel, stained with ethidium bromide, photographed and visible under UV light. The amplicon was later purified and the purified preparation was sanger sequenced at Inqaba biotec west Africa limited (IBWA), Ibadan, Nigeria.

Sequence and phylogenetic analysis. For species identification and sequence homology, acquired sequence data was edited using BioEdit v.7.2.5 (Hall, 1999). The isolate was then compared with known potyvirus sequences using the basic local alignment search tool (BLASTn) tool available at the national center for biotechnology information (NCBI). CLUSTALW (Thompson *et al*., 1994) and BioEdit were used to achieve multiple and pairwise alignments. Sequence demarcation tool (SDT) version 1.2 (Muhire *et al*., 2014) was used to compute pairwise sequence comparisons, with the MUSCLE method (Edgar, 2004) as the alignment option. MEGA version 6 (Tamura *et al*., 2013) was used for phylogenetic re-construction with the neighbour-joining method (Maximum composite model). 1000 random replications were used to compute the bootstrap values.

Results and Discussion

Host range and symptomology. Results of the host range tests monitored 30 days after inoculation (DPI) showed that the virus did not produce symptoms in most of the test plants inoculated but showed systemic symptoms in *N. tabacum, A. conyzoides* and *O. gratissimum* (Fig. 1). All infections were confirmed by the use of serology with the symptomless test plants testing negative (Table 1).

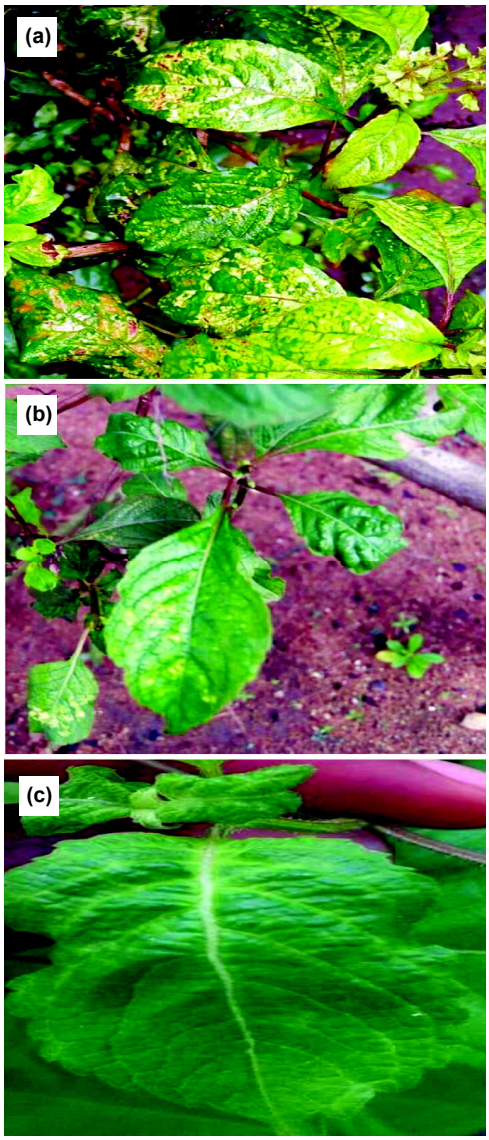

Fig. 1. **(a)** Severe symptoms of viral disease as seen on *O. gratissimum* in the field showing yellow mosaic, **(b)** mosaic symptoms on inoculated *O. gratissimum* and **(c)** mild symptoms observed on test plant in screen-house.

Table 1. Results of host range studies with the virus isolate from *Ocimum gratissimum*

Plant	Infected/inoculated plant	Symptoms	Detection (RT-PCR)
Cucumis sativa	0/10	-	-
Corchorus olitorus	0/10	-	-
Ageratum conyzoides	4/10	M, LM	+
Datura stramonium	0/10	-	-
Ocimum gratissimum	9/10	C, LM, M, VB	+
Nicotiana tabacum	6/10	M, VB	+
Vigna unguiculata	0/10	-	-

+ = present; - = absence; C = chlorosis; M = mosaic; VB = vein banding; VB = vein banding.

Aphid transmission. The virus isolate was transmitted by *A. spiraecola* and *A. craccivora* in a non-persistent manner to 60% and 40% of healthy seedlings of *O. gratissimum* and *N. tabacum*, respectively.

Serology. The *Ocimum* virus isolate reacted positively with the poty virus antisera but negatively against the CMV antisera. The absorbance value of the isolate for the poty virus antibody was 1.607, while for the CMV antibody was 0.582. The positive absorbance value for poty virus was twice greater than that for the healthy control (Table 2).

Nucleotide sequence analysis. The cloned segment contained 697 nucleotides (Fig. 2) of an un-characterised poty virus species (GenBank accession number MT396942) with the gene expressing 232 amino acids. The sequence was compared to other known nucleotide sequences sourced from GenBank using BLASTn analysis. The sequence was found to be homologous to Yam mosaic virus (MG711313). Pairwise sequence analysis revealed 71.1% pairwise identity between the Yam mosaic virus (MG711313) strain and the reference sequence. In addition to the Yam mosaic virus, the Barleria repens mottle virus (ON854904) shared a significant pairwise identity of 71% with the reference sequence (Fig. 3).

Table 2. Results from antigen coated plate enzyme-linked immunosorbent assay

Sample	Location	OD reading at A_{405mm}	
		CMV	Poty virus
Ocimum	Calabar	0.582	1.607*
Healthy		0.326	0.405
Infected		2.687	1.894

* = isolate values were considered positive when the optical density reading was twice greater than the absorbance of healthy controls.

Phylogenetic analysis. The phylogenetic analysis revealed three major phylogroups with the partial sequence gene of the ocimum isolate (MT396942) grouped into the second phylogroup, which also included

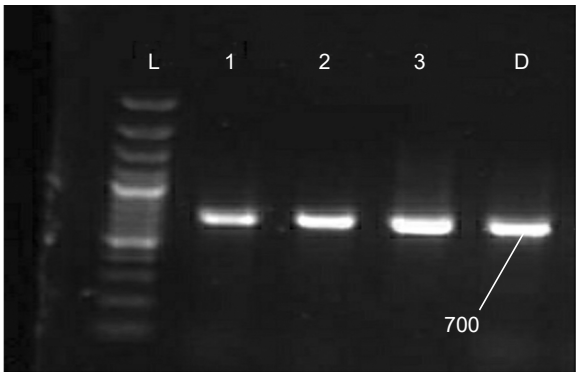

Fig. 2. Amplification for poty virus observed in scent leaf (*O. gratissimum*), DNA size marker (100 bp), Lanes 1-3.

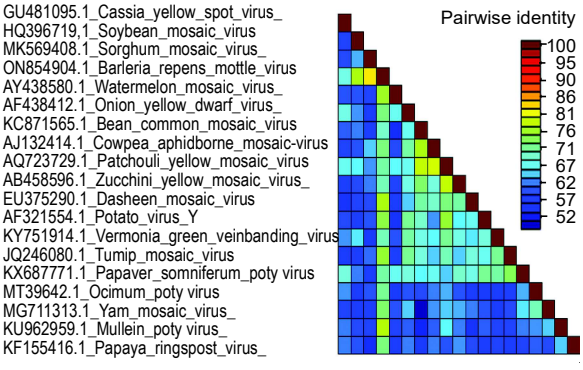

Fig. 3. Comparisons of the ocimum isolate sequence with the poty virus sequence from the genebank, sequences that match to the same species based on a 94% cut-off are coloured the same.

Soybean mosaic virus (HQ396719), Barleria repens mottle virus (ON854904), Sorghum mosaic virus (MK569408), Yam mosaic virus (MG711313), Cassia yellow spot virus (GU481095), Mullein poty virus (KU962959) and Papaya ringspot virus (KF155416). Cassia yellow spot virus (MGU481095) was the closest sequence to the ocimum isolate followed by Barleria repens mottle virus shown in (Fig. 4).

Yellow mosaic on leaves and induced stunting of *O. gratissimum* plants were found in most of the gardens visited. Although this disease had no effect on the plant's seasoning quality, it did add to economic losses and lowered the quality of its essential oils. For optimal disease care, it becomes imperative to identify the virus that causes symptoms. On the basis of host range, the narrow host range observed is consistent with the genus Poty virus (Owolabi *et al*., 2008). Gulya *et al*. (2002) proposed that when a virus has a limited host range, it offers less of a hazard to widely produced crops. The non-persistent transmission of the isolate by aphids suggested that the virus under study could be a poty virus (Eyong *et al*., 2020; Lecoq *et al*., 2001), though viruses from the genus Faba virus, Cucumo virus, Carla virus, Maclura virus and Alfamo virus could also be transmitted in the same way (Ng and Perry, 2004).

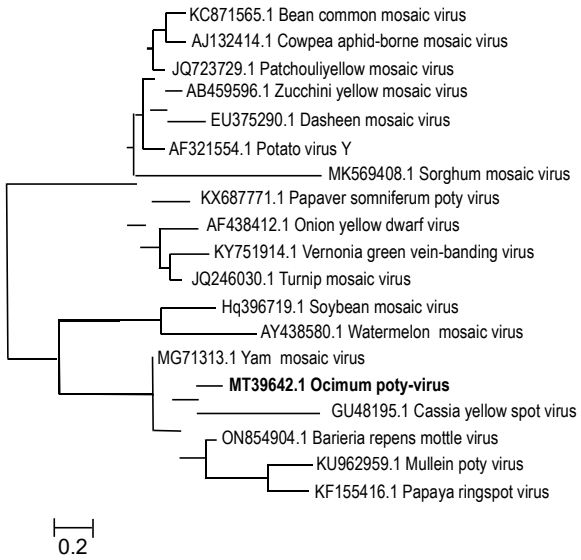

Fig. 4. Phylogenetic maximum likelihood tree reconstructed from nucleotide sequences of *O. gratissimum* poty virus isolate (MT396942) with other related potyvirus sequences with 1000 boots-trap replications.

The positive serological reactivity of the ocimum isolate to poty virus antiserum using a genus-specific ACP-ELISA approach revealed that the virus under investigation was definitely a poty virus. Various publications have described the use of ACP-ELISA for virus genus detection (Arogundade *et al*., 2019; Chen *et al*., 2017; Wulundari and Ermayanti, 2011) and ELISA tests have typically been shown to be cost-effective, quick and accurate (Wangai and Lelgut, 2001).

The molecular characterisation of the virus was studied to further define the species of the isolate after determining its genus. The amplicon size was 697 bp, which corresponded to the expected size of 700 bp when employing a primer specific to the cylindrical inclusion protein of poty viruses. This provided additional evidence that the virus in the study was a poty virus (Pasin *et al*., 2022; Yahaya *et al*., 2019; Ha *et al*., 2008). However, analysis of the nucleotide sequence BLASTn revealed that the sample was only 71.11% identical to the YMV Nigerian isolate (MG711313) and 71 % to the Barleria repens mottle virus (ON854904). Given that the species demarcation threshold for the family Poty-viridae for nucleotide sequences is 76% (Adams *et al*., 2005) and the maximum value found for the partial sequence of the isolate from *O. gratissimum* virus was less than the 76% threshold, the isolate looks to be a different potyvirus and is tentatively referred to as Ocimum mild yellow mosaic virus (OmYMV). Few viruses have been isolated from *O. gratissimum*, including Tobamo virus (Atiri, 1999), CMV (Ekpiken *et al*., 2021a; Sinha and Samad, 2019; Ayo-John and Hughes, 2014) and begomo viruses (Mollel *et al*., 2020). Evolutionary investigations revealed a close link between Yam mosaic virus (MG711313), Barleria repens mottle virus (ON854904), Papaya ringspot virus (KF155416) and Mullein poty virus (KU962959), however it was most closely related to Yam mosaic virus (MG711313). The virus isolate in this study was confirmed to be a poty virus species and not any of the previously identified viruses.

Conclusion

The virus associated with yellow mosaic, leaf malformation, vein banding and stunting on *O. gratissimum* was isolated and identified. Being a perennial plant but there is a chance and it will become a reservoir for plant viruses. Since the study and report of a poty virus infection on *O. gratissimum* was based on CI genes, it is suggested that further studies involving the use of coat protein gene be made. A complete genome sequence of

the virus is also necessary to fully understand its aetiology so that the virus can be better managed.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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