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Mixed infection of a potyvirus and cucumber mosaic virus on *Ocimum gratissimum* in Okoyong and Boje, Cross River State, Nigeria

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Abstract

Viruses have been known to cause considerable yield losses in *Ocimum gratissimum* (L.), a leafy vegetable commonly known as African basil but locally referred to as "scent leaf". The crop is cherished for its essential oil, medicinal and agricultural benefits. Virus-like symptoms on leaves causing mosaic, yellowing, leaf malformation and stunting on *O. gratissimum* (L.) were observed in several farms during the 2018/19 crop season in two locations of Boje and Okoyong in Cross river state. Serological and molecular methods were used to detect viruses infecting the crop at the two locations. Preliminary studies showed the virus could be transmitted mechanically, affirming it was a viral infection. Symptomatic leaf samples from both locations tested positive in antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) to antibodies of genus-specific *Cucumber Mosaic Virus* (CMV) and *Potyvirus.* Primers specific to the coat protein of CMV and the cylindrical inclusion (CI) protein of potyvirus were used for detection of the viruses in reverse transcriptase-polymerase chain reaction (RT-PCR). Obtained sequences were compared to corresponding sequences in the GenBank using the BLASTn program, and the ocimum isolates showed nucleotide sequence similarities in between 89.20 -97.98 % to ocimum potyvirus (MT396942) from Calabar in both locations and two of the collected samples from Okoyong showed a sequence similarity of 100 % with Ocimum CMV isolate from India MN481937. This is the first evidence of potyvirus infection in both locations and mixed infection of CMV and potyvirus on *O. gratissimum* in Nigeria.

Key words: ACP-ELISA, CMV, RT-PCR, Boje, Potyvirus, Okoyong, *Ocimum gratissimum*

Introduction

There has been various reports on viruses infecting *Ocimum* spp., a genus of leafy vegetables belonging to the Lamiaceae family (Nagai *et al.,* 2011; Poojari and Naidu 2013; Chen *et al.,* 2014). There has however not been a documented report on mixed infections occurring in *Ocimum* spp. in general or *Ocimum gratissimum* (L.) in particular. In Nigeria, *Ocimum yellow mosaic virus* has been reported on *O. basilicum* (Atiri, 1999) but none on *O. gratissimum*. The existence of more than one virus in a single plant resulting in varied symptoms at the same time is known as mixed infection (Singhal *et al.,* 2020). Such infections of plant viruses have been reported severally on naturally growing plants and their cultivated counterpart worldwide (Falk and Bruening, 1994). Mukasa *et al.* (2006) reported that when there is a mixed viral infection on plants, it may lead to increased symptom severity and a higher virus titre resulting in a phenomenon referred to as synergistic disease.

Cucumber mosaic virus (CMV), a species in the genus cucumovirus (family - Bromoviridae) is cosmopolitan and infects over 1200 plant species in over 100 families. Zitter and Murphy (2009) classified CMV isolates into subgroups I and II based on serology and nucleotide sequence identity. The virus belonging to subgroups IA and IB has been identified in Nigeria (Eni *et al.,* 2013; Ayo-John and Hughes, 2014; Arogundade *et al.,* 2019a; Adediji, 2019) and is reported to be transmitted through grafting, seeds, mechanical inoulation and aphids in a nonpersistent manner.

Viruses belonging to the genus Potyvirus, family Potyviridae are regarded as the largest genus of plant viruses. They are also transmitted mechanically, through seeds and by aphids in a nonpersistent manner (Simmons and Munkvold, 2014; Gadhave *et al*., 2020; Sevik and Balkaya, 2015; Chatzivassiliou *et al.,* 2016). They have been implicated to causing diseases in cucurbits and other plants but till date there has been no report of its infection in *O. gratissimum*.

The *O. gratissimum* under study exhibited multiple symptoms such as stunting, mosaic, leaf deformation and chlorosis. The objective of this study was to detect the virus (es) infecting this leafy vegetable using both serological and molecular methods in the mangrove and rainforest vegetative zones of Cross river state, Nigeria in south and central parts of the state respectively (Ukeh, 2007).

Materials and methods

Location: A global positioning system was used for the exact location of the sampling sites. Okoyong in Odukpani local government area in Southern Cross river state is on latitude 5° 06' 26" N and longitude 8° 10' 48" E. It has an annual rainfall range of 2000-4300 mm, average temperature of 26° C and an altitude range of 0-100m (Unuigbe *et al.* 2015). Boje, in Boki

local government area in central Cross river state is located on latitude 6° 15' 23" N and longitude 8° 54' 59" E (Takon and Amalu, 2013). Its elevation is between 100-200m above sea level and an average temperature of 27 °C. It also has annual rainfall range of 1300-3000 mm.

Virus isolate: Five leaves of *O. gratissimum* showing symptoms characteristics of viral infection were collected randomly each from twelve sites in Okoyong and twelve sites in Boje. These were stored on silica gel and kept at 4 °C ahead of serological and molecular analysis.

Serology: To determine the genera to which the virus isolates belonged, antigen coated plate enzyme-linked immunosorbent assay (ACP-ELISA) was used as described by Kumar (2009). A polyclonal antiserum specific against *Cucumber mosaic virus* (CMV) was purchased from Agdia Incorporated, USA and potyvirus genus–specific antibodies (AS-573/1; DSMZ, German Resource Centre for Biological Material, Braunschweig, Germany). Viruliferous sap of 100 µL was obtained by grinding 0.1 g of leaf sample *O. gratissimum* in 1 mL of carbonate coating buffer (0.015M Na_2CO_3, 0.0349M $NaHCO_3$, distilled H_2O) and was dispensed into each well of the microtitre plate. After incubation at 37 °C for 1 hour the plate was washed 3 times with a Phosphate buffered saline - Tween (PBS-Tween) with 3 min between each wash. Cross adsorption, used to minimize non-specific reaction due to the presence of antibodies to host plant antigen, was carried out 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 monoclonal antisera were diluted at 1:3000 in the adsorption solution and 100 µL of each monoclonal antiserum diluted in the adsorption solution, was added to ELISA plate and again incubated at 37 °C for 1 hour. After incubation, ELISA plate was washed 3 times with PBS-Tween. Using the alkaline phosphatase (ALP) system, 100 µL of 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 plate incubated at 37 °C for 1 hour. The plate was washed 3 times with PBS-T. After washing, 100 µL of 0.001 g·mL^{-1} of *p*-nitrophenyl phosphate (PNPP) 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 hour. 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 negative control while virus infected plants were used as positive controls. After 1 hour, absorbance was measured at $A_{405\ nm}$ in a BIO-RAD multiscan ELISA reader (ELx 800, Universal Microplate Reader, USA). The samples were considered positive for either CMV or potyvirus when the optical density values obtained was twice the readings of the corresponding negative controls (Kumar, 2009).

RNA extraction, electrophoresis and sequence analysis: Total RNA was extracted from approximately 0.1 g of leaf samples of symptomatic *O. gratissimum* plants using a cetyltrimethylammonium bromide (CTAB) protocol as described by Abarshi *et al*. (2010). The RNA obtained from infected *O. gratissimum* was converted into a complementary DNA (cDNA) before amplification by RT-PCR as described by Pappu *et al.* (1993). The synthesized cDNA was then amplified using a cylindrical inclusion gene set of primers for potyvirus and a universal CMV pimer. The primer pair CMV F4/ CMV R4 (Arogundade *et al.,* 2019b) and CIF/ CIR (Ha *et al.* 2008) were used for further identification of CMV and potyvirus, respectively (Table 1).

Table 1. Nucleotide primers used for CMV and potyvirus detection

Primers	Sequence (5'- 3')	Product size
CMV F4	GCCGTAAGCTGGATGGACAA	497 bp
CMV R4	CCGCTTGTGCGTTTAATGGCT	
CIF	GGIVVIGTIGGIWSIGGIAARTCIAC	692 bp
CIR	ACICCRTTYTCDATDATRTTIGTIGC	

Amplification was carried out in a GeneAmp 9700 PCR system thermocycler (Applied Biosystem Incorporated, USA) using the following thermocyclic conditions for potyvirus: 42 °C for 30 min for reverse transcription, 94 °C for 3 min for initial denaturing, followed by 40 cycles of denaturing at 94 °C for 30 seconds, an annealing step at 40 °C for 30 seconds, an extension at 68 °C for 1 min, and a final extension at 72 °C for 10 min ending the RT-PCR reaction and 42 °C for 30 min for reverse transcription, one cycle consisting of 94 °C for 5 min, followed by 36 cycles of consisting of 94 °C for 30 seconds, then 55 °C for 30 seconds, and 72 °C for 30 seconds, and a final extension at 72 °C for 5 min ending the RT-PCR reaction for CMV.

The PCR reaction products were separated on 1.5 % agarose gel, stained with ethidium bromide, visualized under UV light and photographed. The amplicon were purified by adding 95 % ethanol to 40 µL of the amplicon in new 1500 µL sterile tube and the solution kept in – 80 °C for 10 mins. The tube was centrifuged at 12000 rpm for 10 min and the supernatant discarded. 500 µL of 70 % ethanol was again added to it and centrifuged again 5 mins. The supernatant was discarded and the tube 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 obtained was then sequenced at Inqaba Biotec West Africa Limited, Ibadan (IBWA).

Sequence analysis and phylogeny: Sequence obtained were compared to known viral sequences using basic local alignment search tool (BLASTn) program available at the National Centre for Biotechnology Information (NCBI) and sequence homology established. Multiple alignment was performed using CLUSTALW (Thompson *et al.,* 1994). Phylogenetic relationships were analysed using neighbour joining method (P-distance) with MEGA version 6 (Tamura *et al*., 2013). Bootstrap values were calculated using 1,000 replications.

Results

Symptoms: Samples of the leaves obtained from the field showed yellowing, mosaic, rugosity, severe stunting and malformation (Fig. 1(a-d))

Serology: Twenty of the twenty four *Ocimum* virus isolate samples reacted positively against the potyvirus antisera while two of the twenty four was found to positively react with the CMV antisera. All of the twelve samples from Okoyong reacted positively with the potyvirus antisera while two of the twelve reacted positively to the CMV antisera. None of the twelve *Ocimum* isolate samples from Boje reacted positively to the CMV while eight were found to have reacted positively with the

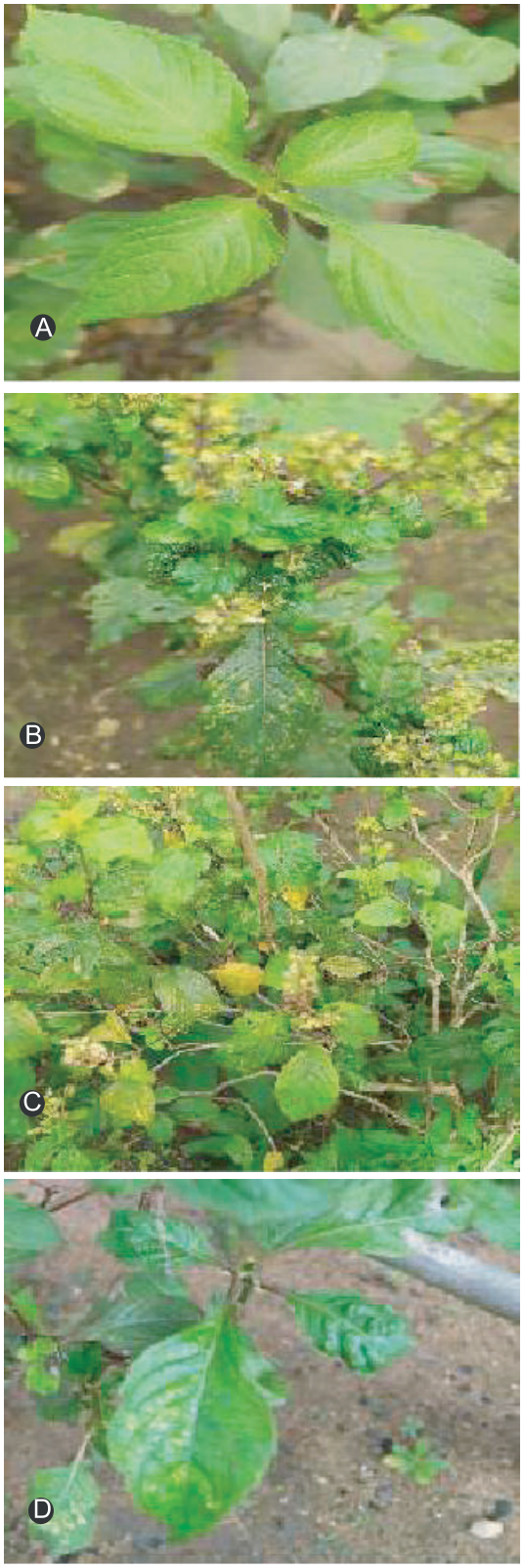

Fig. 1. Symptoms of virus infection in the field (A) healthy leaves of *O. gratissimum,* (B) stunting and malformation, (C) mosaic and yellowing of leaves, (D) rugosity

potyvirus antisera. The absorbance values of the isolate that was found to be twice greater than the value of the healthy control indicated the presence of either CMV or potyvirus (Table 2).

Molecular detection of potyvirus: A 687 bp fragment of CI gene of the infected tissue was amplified. Twenty of the twenty four samples from Boje and Okoyong were amplified representing an

Table 2. Enzyme-linked immunosorbent assay tests results from antigen coated plate showing absorbance value involving virus isolates

Sample	Location	OD reading at A_{405nm} against virus polyclonal antibodies	
		CMV	Potyvirus
1	Boje	0.463	0.338
2	-	0.538	0.563
3	-	0.395	1.607
4	-	0.401	1.838
5	-	0.405	1.653
6	-	0.582	1.521
7	-	0.370	1.486
8	-	0.436	0.982
9	-	0.551	0.521
10	-	0.334	1.112
11	-	0.511	0.991
12	-	0.486	0.318
13	Okoyong	0.397	1.270
14	-	0.497	0.986
15	-	0.501	1.749
16	-	0.529	1.485
17	-	2.394	1.053
18	-	0.409	1.006
19	-	1.230	1.320
20	-	0.395	1.847
21	-	0.459	1.105
22	-	0.486	1.007
23	-	0.562	1.780
24	-	0.430	1.885
Healthy (-)		0.326	0.405
Infected (+)		2.687	1.894

*Values of virus isolates was considered virus positive when the OD reading at A_{405nm} was twice greater than the absorbance from healthy controls

83.3 % positivity for potyvirus with eight of twelve in Boje and twelve of twelve in Okoyong. The presence of potyvirus in the two locations was confirmed (Fig. 2).

Phylogenetic analysis of potyvirus: Ten ocimum isolate from Boje and Okoyong were selected for sequencing and showed sequence homology of 89.20 % - 97.98 % to Ocimum potyvirus (MT396942) (Fig. 3).

Molecular detection of CMV: RT-PCR using the total RNA from infected ocimum sample and gene specific primers for coat protein of CMV (Table 1) resulted in the expected amplicons of ~ 497 bp in samples 17 and 19 from Okoyong (Fig 4). None of the samples from Boje was amplified. The positive amplification for expected size bands indicated an infection by CMV. The amplicon was later sequenced and compared to other known sequences in the GenBank using NCBI BLASTn program.

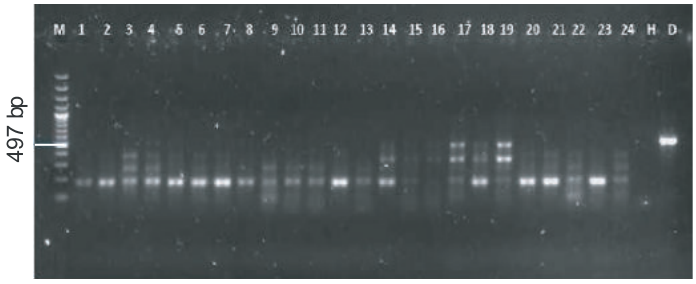

Fig. 2: Detection of CMV gene in *O. gratissimum* leaves using RT-PCR. M = 100 bp DNA; ladder. Lanes 1- 12 = Boje; 13 -24= Okoyong. H = Negative control and D = Positive control

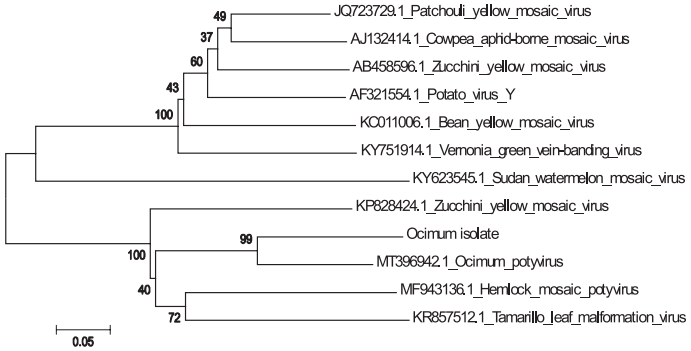

Fig. 3. Phylogenetic analysis of the CI gene of the ocimum isolate with aligned nucleotide sequence generated using neighbor -joining method

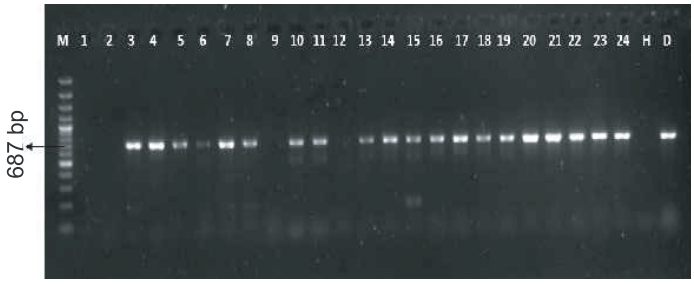

Fig. 4. Detection of potyvirus gene in *O. gratissimum* leaves using RT-PCR. M = 100bp DNA lladder. Lanes 1- 12 ocimum samples from Boje; lanes 13 – 24 ocimum samples from Okoyong. H = Negative control and D = Positive control

Phylogenetic analysis of CMV: The pairwise comparison of these sequences with similar nucleotide sequences from the GenBank using NCBI BLASTn program showed a sequence similarity of 100.00 % to CMV isolate MN481937 from India. The phylogenetic tree produced four clades with AF127976, AB006813 and Z12818 belonging to CMV subgroup II forming the first clade. AJ276481, JQ894819, AB369272 and AM114273 belonged to subgroup IA while DQ412732, AF013291, AM183116, MH260401 and EF178298 belonged to CMV subgroup IB formed the second and third clades respectively. The fourth clade showed the relationship between the ocimum isolate and MN481937 (Fig. 5).

Discussion: Potyviruses and CMV has been reported to infect various crop host all over the world. Both viruses have also been reported to occur naturally as mixed infections (Wang *et al.,* 2002; Fattouh, 2003; Barbosa *et al.,* 2016; Salehzadeh, 2018) in cucurbits resulting in synergistic effect where symptoms are more severe than plants that are singly infected by either of the viruses (Fajinmi, 2019). The study of viruses infecting *O. gratissimum* in Boje and Okoyong both in Cross river state has demonstrated that a strain of the *Ocimum* potyvirus (MT396942) previously deposited with NCBI GenBank, infected the plant samples from both Boje and Okoyong while there was a case of mixed infection of CMV and *Ocimum* potyvirus in two of the twelve samples from Okoyong with the CMV being a strain of MN481937. To our knowledge, this is the first report of mixed infection on *O. gratissimum* from Cross river state, Nigeria. As a single infection, CMV (Sinha and Samad, 2019) and begomoviruses (Mollel *et al.,* 2020) has been reported to infect *O. gratissimum* however no infection by potyvirus has been reported. The ability of potyviruses and CMV to be transmitted by seeds (Ali *et al.,* 2010; Simmons *et al.,* 2013; Hajimorad *et al.,* 2018) and by aphids (Dheepa and Paranjothi, 2010; Gadhave *et al.,* 2020) provide the reasons why they are regarded as cosmopolitan. The implication of mixed infection on *O. gratissimum* is that it can serve as a reservoir for the infection of other crops cultivated in nearby fields. Due to the emergence of novel viruses from frequent mutation, recombination and reassortment of viruses which constantly threatens susceptible economic crop host, it becomes important that further studies on the interaction of the vectors, viruses and host crop be made. This necessitates research into effective, sensitive and quick diagnostic tools as critical for management of plant viruses.

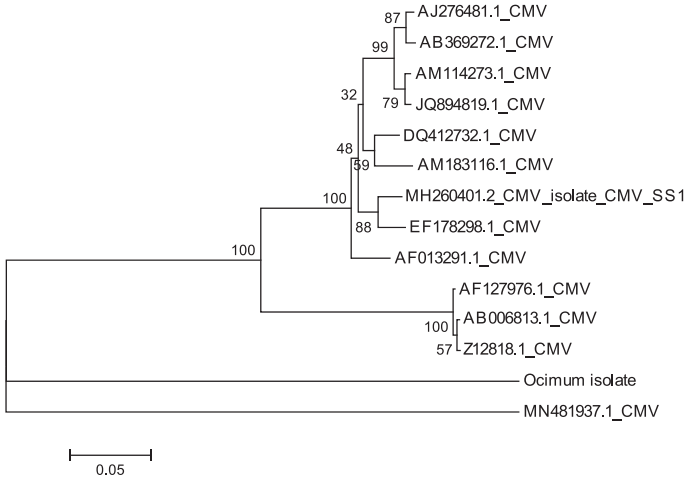

Fig. 5. Phylogenetic analysis of the CP gene of the ocimum isolate with aligned nucleotide sequence generated using neighbor -joining method and MEGA6 software based on Clustal W alignment of the ocimum isolate and other CMV.

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