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Detection and distribution of seed-borne viruses on commercial cowpea (*Vigna unguiculata* (L.) Walp) cultivars in Ado-odo Ota, Ogun Sate, Nigeria

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ABSTRACT

Cowpea is a major source of dietary protein in the nutrition of people in sub-Saharan Africa. Many viruses have been reported to cause economic reduction in cowpea productivity in Nigeria. However, their current distribution is not well documented while most farmers source their seeds from local markets. This study investigated the detection and distribution of seed-transmitted viruses on commercial cowpea cultivars in Ado-odo Ota Local Government area (LGA) of Ogun State. Cowpea seed samples were collected from eleven markets in the LGA. Coordinates of the locations were recorded with Geographical Positioning System (GPS). Thirty cowpea seeds from each market were sowed in planting pots under screenhouse conditions and seedlings were observed weekly for visible virus symptoms. Four-week old seedlings were tested for viruses using Antigen Coated Plate-Enzyme-Linked Immunosorbent Assay (ACP-ELISA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Samples were tested for seven seed-transmitted viruses namely *Blackeye cowpea mosaic virus* (BlCMV), *Cowpea aphid-borne mosaic virus* (CABMV), *Cucumber mosaic virus* (CMV), *Southern bean mosaic virus* (SBMV), *Cowpea mottle virus* (CMeV), *Cowpea yellow mosaic virus* (CYMV) and *Cowpea mild mottle virus* (CPMMV). Seed germination rates ranged from 63.3±15.3% of seeds from Joju to 96.7 ±5.8% for Atan and Iju markets. All samples tested negative to ELISA and were confirmed negative by RT-PCR. These findings suggest absence of seed-transmitted cowpea viruses in the study area as at the time of sampling. Periodic detection survey is recommended to assure the virus-free status of the area.

Keywords: ACP-ELISA, Cowpea viruses, Diagnostics, RT-PCR, Seed transmission

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Introduction

Cowpea *(Vigna unguiculata* L. Walp) is one of the most important leguminous food crops in sub-Saharan Africa. It provides a significant portion of the dietary protein, playing an important nutritional role to the people in developing countries of the tropics and subtropics, especially in sub-Saharan Africa (SSA) (Kareem & Taiwo, 2007). Nigeria is reputed the world's largest producer of cowpea with about 40 % (2.14 million) of the world total (5.59 million tonnes) production (FAO, 2016). Cowpea seed protein content ranges from 23 to 32 % of seed weight, rich in lysine and tryptophan and a substantial amount of mineral and vitamins (Hall et al., 2003). It is a staple food crop in Nigeria (Olakojo et al., 2007) where it serves as an important source of protein for the teeming population. Farmers in the dry savanna use cowpea haulms as a nutritious fodder for their livestock. Its ability to fix atmospheric nitrogen helps maintain soil fertility (Singh et al., 1997).

However, cowpea productivity is low in Nigeria, with an average seed yield of about 577.6 kg ha^{-1} (FAO, 2016). This is due to

infestation by insect pests, parasitic weeds and infections by bacteria, nematodes, fungi, and viruses (Singh, 2014). Viral diseases are important production constraint in Nigeria (Thottappilly and Rossel, 1992), causing serious economic yield losses (10 - 100 %) in cowpea (Rachie, 1985). Natural cowpea infection with about 140 viruses has been observed globally (Hughes and Shoyinka, 2003), of which nine had been reported to occur in SSA, all of which also occur in Nigeria (Taiwo, 2003). These are *Cowpea aphid-borne mosaic virus* (CABMV), genus *Potyvirus*; *Cowpea yellow mosaic virus* (CYMV), genus *Comovirus*; *Black eye cowpea mosaic virus* (BlCMV), genus *Potyvirus*; *Cowpea mottle virus* (CPMoV), genus *Carmovirus*; *Southern been mosaic virus* (SBMV), genus *Sobemovirus*; *Cucumber mosaic virus* (CMV), genus *Cucumovirus*; *Cowpea mild mottle virus* (CPMMV), genus *Carlavirus*; *Cowpea golden mosaic virus* (CPGMV), genus *Begomovirus* and *Sunhemp mosaic virus* (SHMV), genus *Tobamovirus* (Hughes and Shoyinka, 2003; ICTV, 2012). Most of the viral diseases of cowpea lead to overall stunting, reduction in leaf size, mottling, mosaic, leaf chlorosis, leaf distortion, leaf curling, vein clearing, necrotic local lesion and plant death (Akinjogunla, 2005). Seed transmission of cowpea viruses has been previously reported in Nigeria (Hampton et al., 1997). These seed-borne viruses have been reported to have devastating effect on cowpea production causing stunting and plant deformation in early growth stage and not allowing the plants to reach their full potential (Booker et al., 2005; Hampton et al,. 1997). For instance, SBMV was reported to be seed-borne at 3 – 4 % (Thottappilly and Rossel, 1988) while 30 % CMV transmission rate was reported in cowpea (Abdullahi et al., 2001). Cowpea aphid borne mosaic virus CABMV), was also observed to have 0 – 40 % seed transmission in cowpea (Aboul-Ata et al., 1982).

Survey of cowpea viruses conducted in 1991 - 1993 throughout all agro-ecological zones in Nigeria indicated occurrence of six viruses (CABMV, BlCMV, CYMV, BlCMV, SBMV and CMV), five of which were found in some states including Ogun state (Shoyinka et al., 1997). Also, the seven seed-borne viruses considered most damaging to cowpea in Nigeria include BlCMV, CABMV, CMV, CYMV, SBMV, CMeV and CPSMV (Taiwo, 2003). However, there is limited information on current incidence of the viruses and updates of their distribution in the different States of the country. Majority of Nigerian farmers obtain their cowpea seeds for planting from the markets while most of the cowpea viruses have been reported to be seed transmitted. The viruses are further transmitted and spread through insect vectors (Hampton et al., 1997). A high incidence of cowpea pests such as aphids, whitefly and beetles have been reported in cowpea field in Nigeria (Singh et al., 2003) and these pests are known vectors of major viruses (ICTV, 2012). To prevent reduction in cowpea productivity by infection or multiple infections of viral diseases, updated knowledge of their occurrence and distribution are required for effective management strategies while accurate diagnosis using serology assisted with molecular techniques are important in the disease management. This study thus aimed at investigating the current distribution of seed-transmitted viruses on commercial cowpea cultivars in Ado-odo Ota local government area (LGA) of Ogun state.

Materials and method

Sample collection and planting

Cowpea seed samples were randomly collected from eleven markets in Ado-odo Ota LGA of Ogun State (Table 1) between December, 2016 and February, 2017. Coordinates of sample locations were recorded with the aid of a Geographical Positioning System (GPS). Two improved cowpea breeding lines (IT98K-1092-1 and IT99K-1060) obtained from the Cowpea Breeding Unit of IITA, Ibadan, were used as a healthy check. Seedlings were raised in the screenhouses of the Nigeria Agricultural Quarantine Service (NAQS), Moor Plantation, Ibadan under chemical pest control with 'Cyperforce' (Cypermethrin 10 % EC) at 5 ml per litre to guard against insect vectors transmission of viruses. Thirty seeds were sown per sample, 10 seeds per planting bags each containing 7 kg sterilized top soil. Data were taken on germination percentage 6 days after sowing. Seedlings were observed visually for symptoms of seed transmitted viruses on weekly basis for four weeks. Watering was carried out manually every other day while weeding was also performed.

Table 1: Some characteristics of the commercial cowpea

Source Market	Coordinates Lat	Long	Local name /variety	Seed coat colour	Seed texture	Seed size
Iju	6.6806	3.1405	Ewa oloyin	Light brown	Rough	Medium
Atan	6.6632	3.1835	Ewa oloyin	Lightbrown	Rough	Medium
Joju	6.7091	3.2383	Oloyin wewe	Light brown	Rough	Medium
Otta	6.7083	3.2406	Ewa drum	Light brown	Rough	Medium
Oju Ore	6.6884	3.2263	Ewa White	White	Rough	Large
Sango	6.6884	3.2265	Oloyin pelebe	Brown	Rough	Medium
Oloko	6.7071	3.2080	Ewa oloyin	Brown	Rough	Small
Iyana	6.6808	3.1835	Ewa drum	Brown	Rough	Medium
Alishiba	6.7083	3.2340	Ewa drum	Light brown	Rough	Medium
Owode	6.7451	3.2150	Oloyin wewe	Light brown	Rough	Small
Ijako	6.7292	3.2181	Ewa drum	Brown	Rough	Large
IITA I	7.4277	3.9995	IT99K-1060	Light brown	Rough	Small
IITA II	7.4277	3.9995	IT98K-1092-1	Black	Smooth	Large

Lat = latitude, Long = longitude

Virus detection by Antigen Coated Plate-Enzyme Linked Immunosorbent Assay (ACP-ELISA)

Leaf samples of four weeks old seedlings were taken to the Virology and Molecular Diagnostics Unit (VMD) laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria for virus detection using ACP- ELISA. Samples were tested using antisera for six seed transmitted cowpea viruses: CMV, SBMV, CMEV, CPMMV, CYMV and BlCMV. The ACP-ELISA was performed as described by Kumar (2009). Test leaves were ground with a mortar and pestle in coating buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g and sodium diethyl dithiocarbamate 10 g in 1 litre of distilled water with pH adjusted to 9.6) at a ratio of 0.1 g ml^{-1} (1:10 w/v) leaf sample to buffer. Hundred microlitres (100 µl) of the extract was dispensed into each well of the ELISA plate. The plate was incubated inside a humid box at 37 ºC for 1 hr. and washed with three changes of phosphate buffer saline-Tween (PBS-T: $Na2HPO_4$ 22 g, KH_2PO_4 4 g, KCl 4 g, NaCl 160 g and 10 ml of Tween-20, made up to 2 litres with distilled water, at pH 7.4), allowing three minutes interval for each wash. The plate was tap-dried on paper towel to drain the wells. The same washing procedure was carried out after each successive incubation step. Appropriate antibody dilution ratio for each virus was used and the antibody and the leaf sap were incubated for 30 mins. One hundred microlitres of this was dispensed into each well of the ELISA plate and then incubated at 37 °C for 1 hr. The plate was washed and alkaline phosphatase (ALP) conjugated anti-rabbit (goat) antibody (Sigma, USA) was diluted using 1 µl anti-rabbit alkaline in 15 ml conjugate buffer and mixed thoroughly. Then, 100 µl of this was dispensed into each well of the ELISA plate and incubated as above. After this, p-nitro phenyl phosphate (PNP) substrate solution was prepared at a concentration of 1 mg ml^{-1} in substrate buffer (10 % diethanolamine in distilled water, at pH 9.8). Then, 100 µl of PNP was added to each well and the plate incubated in the dark for one hr. at room temperature and overnight (approximately 14 hr.) at 4 °C to allow colour development. Lastly, Optical density (OD) values were read at 405 nm using a BIO-RAD Microplate Reader (ELx 800, Universal Microplate Reader). Readings were taken at one hour and overnight at 4 °C. ELISA positive result was determined by ELISA values greater or equal to twice the absorbance value of healthy control (Kumar, 2009).

Virus detection by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Leaf samples were pooled per location and subjected to confirmatory test for BlCMV, CABMV, CMV and CPMMV using RT-PCR. The remaining three viruses were excluded due to

their high antigenicity for ELISA and also to limited facilities. Total nucleic acid were isolated from the leaf tissues of the plants using modified Cetyltrimethyl Ammonium Bromide (CTAB) method according to Abarshi et al., (2010) and stored at -20 ºC before use. Quality of the extracted nucleic acid was analyzed by agarose gel electrophoresis described by Kumar (2009). The nucleic acid fragments were visualized with UV- transilluminator at 302 nm. RNA concentration and purity were estimated with NanoDrop (2000) spectrophotometer (Thermo scientific Tegrant Corporation).

RT-PCR was performed by the procedure described by Kumar (2009) using specific primer pairs (Table 2). The total RNA extracted was diluted in ratio 1:50 and 12.5 µl PCR reaction mixture was prepared which comprised of 10 × reaction buffer (flexi), 0.75µl of 25 mM $MgCl_2$, 0.25 µl mixture of 10 mM dNTPs (NEB), 0.25 µl (10 mM) of respective primers (IDT), 0.06 µl Taq DNA polymerase (5 U ml^{-1}) (Promega Corporation, USA), 0.06 µl M-MLV Reverse transcriptase (200 U ml^{-1}) (Promega Corporation, USA), 2.0 µl of 10 ng/µl genomic DNA and sterile distilled water. PCR amplification was performed with Applied Biosystems (GeneAmp® PCR System 9700) Cycler machine using lyophilized PCR micro tubes (Promega Corporation, USA). Amplification of BlCMV RNA was done using 1 cycle of 42°C (30 min); 1 cycle of 94°C (5 min); 35 cycles of 94°C (30 sec), 55°C (30sec), 72°C (30sec); 1 cycle of 72°C (5 min); and then at 4°C till the end. For CMV, RNA amplification was carried out with 1 cycle of 42°C (30 min); 1 cycle of 94°C (5 min); 35 cycles of 94°C (30sec), 55°C (30sec), 72°C (30sec); 1 cycle of 72°C (5 min); then at 4°C. CABMV was amplified by 1 cycle of 42°C (30 min); 1 cycle of 94°C (5 min); 35 cycles of 94°C (30 sec), 55°C (30 sec) and 72°C (30 sec); 1 cycle of 72°C (5 min) and at 4°C till end. For CPMMV, amplification was by 1 cycle of 42°C (30 min); 1 cycle of 94°C (5 min); 35 cycles of 94°C (30sec) 55°C (30sec) 72°C (30sec); 1 cycle of 72°C (5 min) and at 4°C till the end of the process. The RT-PCR product was analyzed by agarose gel electrophoresis by loading the well with the 12.5 µl amplicons using 4 µl 100 bp DNA marker. PCR was read positive or negative by presence or absence of amplified DNA bands

Table 2: Primers used in RT-PCR

Virus	Primer	Sequence
BlCMV	BCMV F	ATGTGGTACAATGCTGTGAAG
	BCMV R	TTTCAGTATTCTCGCTGGTTG
CABMV	CABMV F	GTACTCCAGTCTGATGGAAAGG
	CABMV R	GTCCGAGAAGTGGTGCATAA
CMV	CMV F	GCCGTAAGCTGGATGGACAA
	CMV R	CCGCTTGTGCGTTTAATGGCT
CPMMV	CPMMV 1	CACTTGGAATTTTATGTTGAC
	CPMMV 2	TCATTTCGATTGGACCTATC

Data collection and analysis

Data were collected on percentage seed germination, virus titre value (ELISA value read at 405nm Absorbance) and PCR scores. Seed germination data were analysed by analysis of variance using SAS software (SAS, 2008) with means separated by Duncan Multiple Range Test (DMRT) at p = 0.05.

Results

Characteristics of cowpea seed samples

Characteristics of the collected seed samples are presented in Table 1. The seed coat colour ranged from white to light brown, brown or black. Seed coat texture was rough for all the seeds except the smooth textured seeds from IITA while the seed sizes ranged from small to large.

Seed Germination

All the cowpea seeds germinated from 5 to 6 days after sowing. There were significant differences (p = 0.05) in the rate of seed germination (Table 3). Germination was low (below 90 %) in seed samples from Joju, Oju Ore, Alishiba, Owode and Ijako markets but higher in seeds from other locations. Germination rate ranged from 63.3 ± 15.3% for Joju to 96.7 ± 5.8% for both Atan and Iju markets as well as IITA II.

Table 3: Seed germination (%) of cowpea at six days after planting

Cowpea source	Germination (%)
Iju	96.7± 5.8a
Atan	96.7±5.8a
Joju	63.3±15.3c
Otta	93.3±11.6ab
Oju Ore	86.7±5.8ab
Sango	90.0±10.0ab
Oloko	93.9±11.6ab
Iyana	93.3±11.6ab
Alishiba	73.3±15.3bc
Owode	76.7±20.8abc
Ijako	73.3±5.8bc
IITA I (check)	90.0±0.0ab
IITA II (check)	96.7± 5.8a

Values are means ± Sd. Means followed by the same letter are not signiucantly different (p = 0.05) by Duncan Multiple Range Test

Assessment of seed transmitted viruses

Weekly visual examination of the seedlings for symptom for a period of four weeks showed lack of virus symptom on all the cowpea seedlings. Although mild chlorosis was observed from seedlings from Owode market, the symptom disappeared after two weeks. Negative serological result was also observed as all the samples tested negative to the six cowpea viruses (BlCMV, CMV, SBMV, CMeV and CPPMV) by ACP-ELISA at both one hour and overnight (14 hours) readings (Table 4). Similarly result was observed from the confirmatory test of all the samples for BlCMV, CABMV, CMV and CPMMV using RT-PCR (Figure 1).

Table 4: Virus test of cowpea using Enzyme Linked Immunosorbent Assay (ELISA)

Cowpea	BlCMV		CMV		SBMV		CMeV		CYMV		CPMMV	
Source	1 hr	14 hr	1 hr	14 hr	1 hr	14 hr	1 hr	14 hr	1 hr	14hr	1 hr	14 hr
Iju	0.13	0.30	0.13	0.30	0.09	0.17	0.11	0.14	0.14	0.31	0.10	0.17
Atan	0.13	0.30	0.13	0.28	0.12	0.17	0.12	0.12	0.17	0.34	0.10	0.15
Joju	0.13	0.33	0.13	0.30	0.14	0.17	0.11	0.13	0.15	0.32	0.10	0.15
Otta	0.12	0.28	0.13	0.30	0.08	0.15	0.10	0.12	0.17	0.38	0.10	0.15
Oju Ore	0.12	0.29	0.12	0.25	0.12	0.15	0.09	0.13	0.14	0.32	0.10	0.17
Sango	0.13	0.34	0.13	0.30	0.09	0.10	0.10	0.11	0.17	0.28	0.10	0.14
Oloko	0.12	0.30	0.12	0.30	0.12	0.16	0.10	0.12	0.17	0.33	0.10	0.12
Iyana	0.11	0.24	0.11	0.30	0.10	0.20	0.09	0.10	0.16	0.35	0.11	0.15
Alishiba	0.18	0.21	0.20	0.16	0.14	0.17	0.15	0.16	0.19	0.37	0.10	0.15
Owode	0.12	0.21	0.11	0.16	0.08	0.15	0.09	0.13	0.24	0.42	0.11	0.17
Ijako	0.11	0.18	0.10	0.12	0.09	0.11	0.09	0.10	0.14	0.29	0.09	0.13
IITA I	0.13	0.33	0.13	0.30	0.10	0.14	0.09	0.11	0.19	0.41	0.11	0.15
IITA II	0.12	0.31	0.12	0.29	0.09	0.11	0.10	0.10	0.16	0.34	0.12	0.19
Disease	0.52++	1.65++	0.91++	2.88++	0.78++	2.36++	0.65++	2.03++	0.44+	1.24++	0.61++	1.30++
Healthy	0.16	0.19	0.15	0.16	0.11	0.14	0.11	0.11	0.17	0.25	0.14	0.15
Buffer	0.17	0.17	0.14	0.14	0.12	0.15	0.12	0.13	0.14	0.16	0.13	0.19

BlCMV, *Blackeye cowpea mosaic virus*; CMV, *Cucumber mosaic virus*; SBMV, *Southern bean mosaic virus*; CMeV, *Cowpea mottle virus*; CYMV, *Cowpea yellow mosaic virus*; CPMMV, *Cowpea mild mottle virus*; 1 hr, one hour reading(ELISA value read at 405nm Absorbance); 14hr, fourteen hour (or overnight) reading; - = ELISA negative result; + = ELISA positive (ELISA values = 2 x Healthy control); ++ = highly positive (= 3 x Healthy control) result

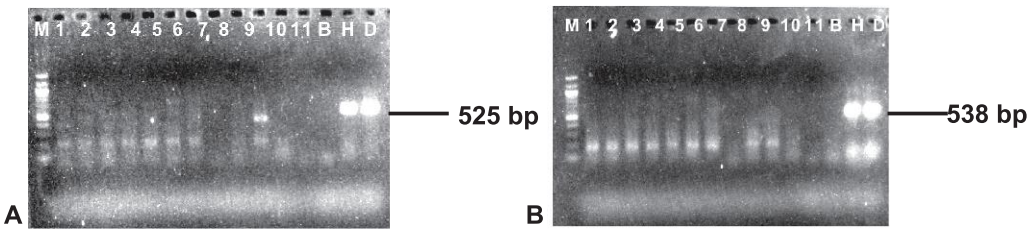


Figure 1. No amplification observed in cowpea plants by RT-PCR using (A) CABMV and (B) CMV specific primers; M, DNA size marker (100 bp; Promega, USA); lanes 1 - 11, extracts from cowpea samples from 11 locations; B = buffer control, H = healthy control, D = positive control.

Discussion

Cowpea is the most important leguminous food crop in West Africa and an important source of protein to the diet of the developing countries (Singh et al., 1997). Viral disease is one of the major constraints to cowpea productivity (Taiwo, 2003). Since most of the cowpea viruses are seed transmitted (ICTV, 2012; Sastry, 2013) and most farmers in Nigeria source their seeds for planting from the markets, commercial cowpea seeds from selected markets in Ado-odo Ota LGA of Ogun state were surveyed for seed transmitted viruses. Most of the collected seed samples were of brown coat colour, medium size and rough textured and they germinated at different rates. Adverse effects of seed transmitted viruses on seed germination have been reported in legumes. Mandhare and Gawade (2010) reported that *Soybean mosaic virus* (SMV) infection caused 18 – 33 % reduction in soybean seed germination. In this study, cowpea seeds from five markets showed poor germination rate of less than 90 % and germination rate varied from 63.3 ± 15.3 % of seeds from Joju to 96.7 ± 5.8 % for Atan and Iju markets. The low germination rate of cowpea seeds from some markets (Oju Ore II, Igbala, Joju, Alishiba and Ijako) may be attributed to poor storage temperature of seed by the market women and exposure of the seeds to sunlight in the markets.

Accurate detection is the first step to viral disease management. Effective use of ELISA and PCR based techniques have been reported in detection of various cowpea viruses (Taiwo, 2003) with PCR methods giving more accurate detection without any false positive reaction as is sometimes observed with ELISA (Akinjogunla et al., 2007). A single diagnostic test or assay may provide adequate information on the virus identity but a combination of methods is generally needed which are specific, sensitive and inexpensive (Naidu and Hughes, 2003). This study shows that the cowpea seed samples collected from eleven markets in Ado-od Ota LGA were apparently free from viruses as all the seed samples resulted in symptomless seedlings which were negative to ELISA and confirmed negative to most of the viruses by RT-PCR. Though occurrence of five cowpea viruses were reported in Ogun state from a nation-wide survey (Shoyinka et al., 1997), seeds from the study area seem to be free of cowpea viruses. This also suggests that the low germination rate in some of the seeds is not connected with viral infections. Such seeds, free from seed transmitted viruses, can be used as planting materials when viable, although farmers are encouraged to source their seed for planting from seed companies, National Agricultural Seed Council, Agricultural Research Institutes and Agricultural Development Programme offices. Seed transmission serves as source of inoculum of viral diseases. Seed-borne cowpea viruses, after establishment in plants, are typically spread within fields by insect vectors such as aphids (e.g. *Aphis craccivora*) (Hampton et al 1997). Hence, seed and insect vector transmissions play important roles in the spread and epidemiology of viral diseases. For example, only 5 % seed transmission of *Zucchini yellow mosaic virus* (ZYMV, genus *Potyvirus*) in oil pumpkin (*Cucurbita pepo* var. *styriaca*) later resulted in a devastating effect causing 99 % yield loss (Riedle-Bauer et al, 2002). This increasingly recognized importance of seed transmission in plant virus ecology has led to the strengthening of seed-health testing for viruses in certification and quarantine agencies internationally. Parts of virus disease management include eradicating the source of infection to prevent the virus from reaching the crop such as, rouging of infected plants to prevent the spread of the viruses, use of plant quarantine, use of resistant varieties and utilizing virus-free planting materials (Khetarpal et al., 1998; Naidu and Hughes, 2003). The use of such virus-free cowpea seeds together with other viral disease management methods will enhance cowpea productivity.

Conclusion

This study shows absence of seven seed transmitted cowpea viruses (BlCMV, CABMV, CMV, SBMV, CMeV, CYMV and CPMMV) from seeds of commercial cultivars in Ado-odo Ota LGAs of Ogun State as at the time of sampling. Although, the seven viruses have been reported in many parts of Nigeria, this finding presents a good starting point for cowpea virus disease management in the study area since most farmers obtain their cowpea planting materials from the markets. However, there is a need for regular virus disease surveillance for update information on the incidence and spread as well

Field survey of cowpea farms is also important to confirm the absence of cowpea viruses in the area.

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