SCREENING OF CASSAVA PLANT IN OYE EKITI FOR AFRICAN CASSAVA (*Manihot esculenta)*Crantz, MOSAIC DISEASE (CMD) INFECTION BY POLYMERASE CHAIN REACTION(PCR)

OMODARA, Oluwapelumi Peter

(BTH/11/0262)

A FINAL YEAR PROJECT SUBMITTED TO THE DEPARTMENT OF PLANT SCIENCE AND BIOTECHNOLOGY, FACULTY OF SCIENCE INPARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE(B.SC)DEGREE IN PLANT SCIENCE AND BIOTECHNOLOGY

OCTOBER, 2015

DECLARATION

I hereby declared that this project has been prepared by me, it a record of my own research work and to the best of my knowledge and has not been published or presented in any form. All sources of information are dully acknowledge by means of references

Omodara Oluwapelumi Peter

Sign .....

October, 2015

CERTIFICATION

This is to certify that this final year project report was carried out and compiled by me OMODARA OLUWAPELUMI PETER with matriculation number BTH/11/0262 in the department of Plant Science and Biotechnology, Faculty of Science, Federal University Oye Ekiti,Ekiti State. Under the supervisor of PROF SLYVIA UZOCHUKWU. All assistance and contribution received have also been acknowledged.

.....
PROF .S.V. A. UZOCHUKWU
(SUPERVISOR)

24/11/15
.....
Date

.....
Dr A.A AJIBOYE
(Head of Department)

24/11/15
.....
Date

DEDICATION

This project is dedicated to GOD almighty.

ACKNOWLEDGMENT

I thank God Almighty for his mercy and grace, I am greatly indebted to my parents, Mr. and Mrs. Omodara for their financial and moral support and understanding during the course of my programme.

My special thanks goes to Prof S.V.A Uzochukwu for her motherly role, also i will love to thank DR M.A Adekoya for her support.

Special gratitude goes to all the staff in the virology and molecular diagnostics unit at international institute of tropical agriculture in Ibadan, especially DR L.P KUMAR,DR .O. Kolade,Mrs P. Ogunsanya,MR D.Oresanya for their support and encouragementthroughout my stay in the laboratory ,and also Mr Dele Ogunfumilayo,and Mr Adewale Kazeem of NIGERIA AGRICULTURAL QURANTINE SERVICES(NAQS)

My special thanks also goes to DR A.A Ajiboye,DR .J.A Agbolade,and other member of staff in the Department of plant science and biotechnology both academics and non-academics, my thanks also extends to the Head of department of GEOPHYSICS DR JEGEDE and the Technologist, I will also need to thanks my siblings Mr T.F Omodara and Miss O.F Omodara ,friends most especially Olanbiwonnu Temitayo David, Omotunde Mercy, Alozie precious, Olaiya Aderonke,Umoru Opeyemi, Adebayo Adebola,and all my departmental course mateand relatives most especially Mr and MRS G.B Onijingin and my lovely GrandparentMR and Mrs Onijingin

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ABSTRACTS

Cassava is vulnerable to a broad range of diseases caused by viruses. Among them, cassava mosaic disease (CMD) is the most severe and widespread, limiting production of the crop in sub-Saharan Africa.CMD-affected cassava plants produce few or no tubers depending on the severity of the disease and the age of the plant at the time of infection.CMD is one of the major constraints for cassava production in Nigeria. This study was therefore undertaken to determine the extent of CMD infestation in Oye-Ekiti, South West, Nigeria. A multiplex polymerase chain reaction (PCR) was carried out using the Oja mix primer mix for the simultaneous detection of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV). Altogether, 60 cassava samples from 6 farms were evaluated. Only 7 samples amplified with ACMV showing that 11.7% were positive to ACMV and no samples amplified with EACMV in single and mixed infections, and sequencing results confirmed virus identities according to the sequences of begomovirus species. By using the multiplex techniques, time was saved and amount of reagents used were reduced, which translated into reduced cost of the diagnostics. This tool can be used by cassava breeders screening for disease resistance; scientists doing virus diagnostic studies; phytosanitary officers checking movement of diseased planting materials, and seed certificationand multipliers, for virus indexing. The study showed that the ACMD load in Oye-Ekiti is not high and that EACMD has not reached the area at all.

CHAPTER ONE

1.0 INTRODUCTION AND LITREATURE REVIEW

Cassava (*Manihot esculenta* Crantz, family Euphorbaiceae, synonyms: yucca, manioc, and mandioca), a native to South America, is believed to have been introduced into sub-Saharan Africa by the Portuguese traders during the 16th century. According to the Food and Agriculture Organization of the United Nations (FAO), cassava is currently the third most important source of calories in the tropics, after rice and corn. More than 800 million people use cassava as a source of food and income generation in Africa, Asia, and Latin America. Among the cassava-growing regions of the world, Africa accounts for more than 50% of the global cassava production of 233.8 million metric tons. The resilience of cassava enables it to grow successfully under a wide range of agro-ecological zones where cereals and other crops cannot thrive, making it a suitable crop for poor farmers to cultivate under marginal environments in Africa. The other attraction for farmers to grow cassava is that it produces higher yields per unit of land than other crops such as yams, wheat, rice, and maize. The pivotal role of cassava in the lives of Africans is evident from many documents.

Cassava is cultivated as a tuberous root crop and its roots are the major source of dietary starch. The tubers are eaten fresh and in various forms of processed food. Cassava is grown in sub-Saharan Africa by resource-poor farmers, many of them women, as an intercrop with vegetables, plantation crops (coconut, oil palm and coffee), yams, melon, sweet potato, maize, sorghum, millet, cowpea, groundnut (peanut), and other legumes for food security and assured household income. Cassava leaves are also consumed as a green vegetable, especially in East Africa, to provide an important source of proteins, minerals, and vitamins. Several African countries are gradually replacing wheat flour with cassava flour in the production of staples like

bread and noodles.

However, there is an increased awareness that excessive reliance on cassava could lead to malnutrition in these countries, since the tubers are a poor source of protein, vitamins A and E, iron, and zinc. In recent years, cassava has been increasingly used as raw material in the manufacture of various industrial products such as starch and flour. With increased prospects of starch from cassava as a source of ethanol for biofuels, its cultivation is transforming from subsistence to a more commercially-oriented farming enterprise. Consequently, cassava has been increasing throughout Africa.

1.1 GEOGRAPHICAL DESCRIPTION OF OYE EKITI

Oye Ekiti is a town and headquarter of Oye Local Government Area in Ekiti State South West, Nigeria, With the longitude and latitude of 5.333333 and 7.8 respectively , The town is mainly an upland zone, rising over 250 meters above sea level. It lies on an area underlain by metamorphic rock, Oye enjoys tropical climate with two distinct seasons. including the rainy season (April–October) and the dry season (November–March). Temperature ranges between 21° and 28 °C with high humidity. The south westerly wind and the northeast trade winds blow in the rainy and dry (Harmattan) seasons respectively. Dominated by savannah grassland.

1.2 CASSAVA MOSAIC DISEASE

Cassava is vulnerable to a broad range of diseases caused by viruses. Among them, cassava mosaic disease (CMD) is the most severe and widespread, limiting production of the crop in sub-Saharan Africa.CMD produces a variety of foliar symptoms that include mosaic, mottling, misshapen and twisted leaflets, and an overall reduction in size of leaves and plants. CMD-affected cassava plants produce few or no tubers depending on the severity of the disease and the

age of the plant at the time of infection.

Cassava mosaic disease in sub-Saharan Africa is caused mainly by African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) (Bock and Woods 1983; Hong *et. al.,* 1993). The two viruses belong to the genus Begomovirus, family Gehbgxminiviridae (Murphy *et. al.,* 1995; Mayo and Pringle, 1998). For 14 years, when ACMV was confirmed as the causal agent of CMD (Bock and Woods, 1983), the virus remained the only known causal agent of the disease in Nigeria until 1997 when EACMV was diagnosed as an additional causal agent (Ogbe *et. al.,*, 1999). ACMV and EACMV had been identified as causal agents of CMD in East Africa, in Kenya and Tanzania, for example (Swanson and Harrison, 1994).

Historically, the first report of CMD came from the Usambaras Mountains range in northeast Tanzania in 1894. The disease was named as "Kräuselkrankheit," a German word that translates to "rippling/crinkling illness" (which describes symptoms observed on affected plants. Although a virus was originally believed to be the causal agent of CMD and that its transmission occurred via whiteflies, it viral status was not proven until the 1970s when small, quasi-isometric, geminate particles, were found in symptomatic host tissues. However, it would not be until many years later that the virus, African cassava mosaic virus (ACMV), was molecularly characterized and Koch's postulates fulfilled.

Although EACMV occurs in Nigeria, the dominant virus is ACMV as observed in a diagnostic survey conducted in farmers' fields in 1997 and 1998

Cassava mosaic virus is the major constraint to cassava production in Nigeria, Cassava plays a key role in the food security of sub-Saharan Africa, but as a vegetatively propagated crop, it is particularly vulnerable to the effects of virus diseases and these therefore represent a major threat to the livelihoods of millions of Africans,

Characteristic symptoms of CMD for plants infected with a single virus range from green to yellow mosaic of affected leaves coupled with leaf distortion, regardless of the infecting CMB species. These morphological alterations in cassava often result in lost tuber yield, and significant storage root yield losses can occur even in resistant genotypes that show only mild or no foliar symptoms .Overall, storage root yield loss across sub-Saharan Africa were estimated between 15 and 24 % annually, which is equivalent to million tons or an annual loss of US$ 1.2 to 2.3 billion.

1.3 THE VIRUSES OF CASSAVA

Cassava is a vegetatively propagated crop, and diseases from viruses cause particular problems as they are carried from one crop cycle to the next through the cuttings used as planting material. Without intervention, infection can therefore readily build up from one crop cycle to the next, particularly where there is also a significant level of vector transmission. Despite the relatively recent arrival of cassava in Africa, there is almost as much diversity among viruses of cassava in Africa as there is in the South/Central American region of origin.

Plate 1: *Bemisia tabaci* on a cassava leaves

The whitefly vector, *Bemisia tabaci* (Hemiptera: Aleyrodidae; plate 1), is mainly responsible for the secondary spread of CMBs although other species of whitefly, such as *Bemisia afer,* can also transmit CMD. Starvation of non viruliferous whiteflies prior to acquisition feeding on infected cassava accelerated the acquisition of ACMV. Once acquired, a latent period of about 6-8 h must lapse before the whitefly is able to transmit the virus, which can thereafter be retained by an infectious whitefly for about 9 days. Viruliferous whiteflies require 10-30 min inoculation access periods for virus inoculation into healthy cassava plants. Under experimental conditions, ten viruliferous whiteflies are needed to achieve the optimal rate of transmission when released on a cassava plant, although a single whitefly is capable of virus transmission.

The presence of African cassava mosaic virus (ACMV) and other cassava mosaic disease (CMD) viruses in cassava (*Manihot esculenta Crantz*) can cause severe damage leading to huge economic loss to infected cassava plants; One of the greatest from infected plants through the use of somatic problems confronting this all important crop in Africa embryogenesis. Parmessur *et. al.,* (2002) reported is Cassava Mosaic Disease (CMD). In Africa, this successful elimination of virus and phytoplasma from disease is caused by the viruses.

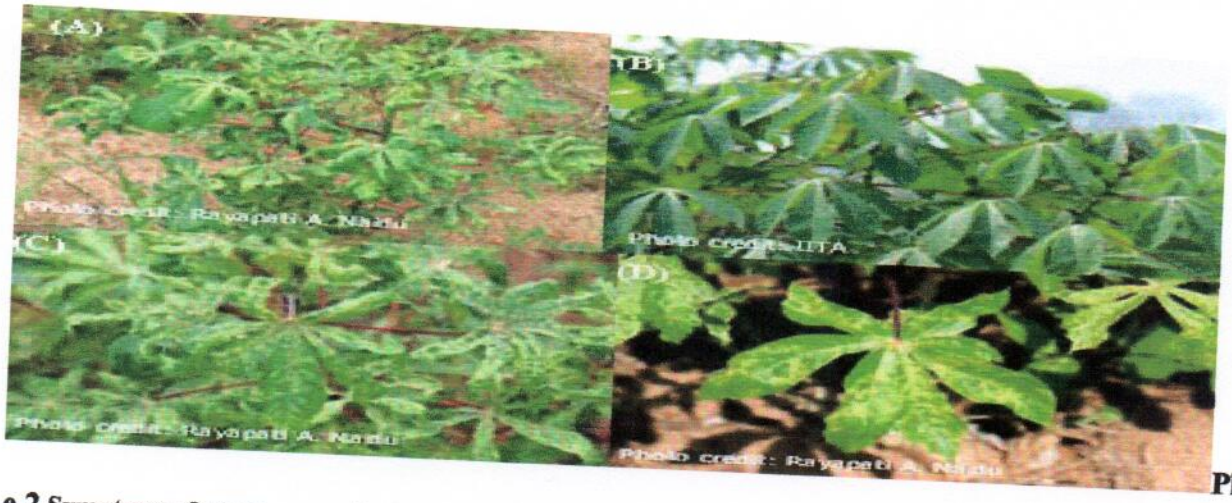


Plate 2 Symptoms of cassava mosaic disease (CMD) on cassava. An infected plant showing severe stunting and distortion of leaves (A) compared to a healthy plant (B). Leaves of CMD-affected plants produce misshapen and twisted leaflets with mosaic and mottling symptoms (C and D).

African Cassava infected with Mosaic Virus (ACMV) and East African Cassava Mosaic Virus (EACMV). They are transmitted by the phytoplasma using the technique of somatic embryogenesis whiteflies (*Bemisia tabaci*), and sometimes through. Infected stem (D' Onghia *et. al.*, 2001) Presence of these viruses can cause losses in somatic embryogenesis using infected stigma and of up to 40% to 50% of total yields in cassava style as explants. Similarly, (Gribaudo *et. al.*, 2006) in throughout the continent

1.4 OBJECTIVES OF RESEARCH

THE BROAD OBJECTIVES

Objectives of this study therefore is to determine the presence and extent of CMD infestation of the virus in Oye Ekiti cassava farm.

SPECIFIC OBJECTIVES

Collection of cassava leaves from farm, to extract the DNA from the cassava leaf, amplify the DNA with the primer sequence to determine which one has the infection, To determine the distribution of cassava mosaic virus in Oye Ekiti

1.5 LITREATURE REVIEW

Cassava plants infected with CMV express a range of symptoms which depend on the virus species/strain, environmental conditions, and the sensitivity of the cassava host. The most typical symptoms consist of a yellow or pale green chlorotic mosaic of leaves, commonly accompanied by distortion and crumpling. Symptoms are readily distinguished from those of mineral deficiency or cassava green mite damage as the virus induced chlorosis and malformation of leaflets is asymmetrical about the midrib. Where symptoms are severe, the plant becomes generally stunted and petioles immediately below the shoot tip may be angled downwards and occasionally may become necrotic, shrivel, and abscise. Where the virus or virus strain is mild or the cassava variety is tolerant, leaf chlorosis may be patchy and absent on some leaves, and there is little or no leaf distortion or malformation and little effect on overall plant vigor. The PCR method of detection is superior because it will detect the presence of the virus even when there are no symptoms (Legg and Thresh, 2003).

The early detection and identification of plant borne pathogens is an integral part of successful disease management and this is especially important in relation to the importation of foreign plant material. The rapid identification of a plant pathogen, allows for the appropriate control measures to be applied prior to the further spread of the disease or its introduction. Since most plant-based foodstuffs have a finite shelf life, it is imperative that any potentially infected

material is diagnosed as rapidly and as reliably as possible, in order to avoid delays and expensive losses to the cash import.

Today, diagnosticians have an array of methodologies to help in this respect. Traditionally, cultural methods have been employed to isolate and identify potential pathogens. This is a relatively slow process, often requiring skilled taxonomists to reliably identify the pathogen. This practice is made all the more difficult due to a number of factors, such as ambiguities in morphological characters, or the specific nutrient requirements & growth conditions of certain pathogens grown in vitro, or time constraints imposed by slow growing pathogens in vitro. However over the last 30 years, several techniques have been developed which have found application in plant pathogen diagnosis; these include the use of monoclonal antibodies and enzyme-linked immunosorbent assay (ELISA), which drastically increased the speed in which pathogen antigens could be detected in vivo, and DNA-based technologies, such as the polymerase chain reaction (PCR) which enable regions of the pathogen's genome to be amplified several million fold, thus increasing the sensitivity of pathogen detection. Despite such advances, cultural diagnosis still predominates, largely due to the technical experience & costs associated with the more recent techniques. Rossel H. W., Thottappilly G., Van Lent J. M. W.,Huttinga H., (1988).

CMD has been reported as occurring at varying levels of incidence throughout the cassava belt of Africa (Ogbe *et. al.*,, 2003b; Thresh *et. al.*,, 1997).

For several years after African cassava mosaic virus (ACMV) was confirmed as the causal agent of CMD, it remained the only known causal agent of CMD in Nigeria until the 1990s when East African cassava mosaic Cameroon virus (EACMCV) and several variants of the EACMV were

diagnosed as additional causative agents (Ogbe *et. al.,* 2003a; 2003b; Ariyo *et. al.,* 2005; Ogbe *et. al.,*, 2006; Alabi *et. al.,* 2008b).

For several years after African cassava mosaic virus (ACMV) was confirmed as the causal agent of CMD, it remained the only known causal agent of CMD in Nigeria until the 1990s when East African cassava mosaic Cameroon virus (EACMCV) and several variants of the EACMV were diagnosed as additional causative agents (Ogbe *et. al.,* 2003a; 2003b; Ariyo *et. al.,* 2005; Ogbe *et. al.,* 2006; Alabi *et. al.,* 2008b).

Eight begomoviruses (genus Begomovirus, family Geminiviridae) have so far been documented in cassava infected with CMD worldwide (Fauquet and Stanley, 2003). Only six of these cassava infecting begomoviruses (CMBs) have been reported occurring in Sub-Saharan Africa. They are: African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), East African cassava mosaic Cameroon virus (EACMCV), East African cassava mosaic Malawi virus (EACMMV), East African cassava mosaic Zanzibar virus (EACMZV) and South African cassava mosaic virus (SACMV) (Fauquetet *et. al.,*, 2008).

Definitive studies confirming that the whitefly *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) transmits CMGs were carried out by Chant (1958) and Dubern (1994). Transmission was shown to be persistent, transracial but not transovarial (Dubern, 1994), with minimum times for acquisition, latent period, and inoculation of 3.5 hours and 5–10 minutes, respectively. Reports on transmission efficiency have varied from very low (0.15–1.7% of individuals infective) for field-collected insects (Fargette *et. al.,*, 1985), to moderate (4–13%) for laboratory-reared insects (Dubern 1994; Maruthi *et. al.,*, 2002). Recent evidence also suggests that there is only limited coadaptation between CMGs and their vector within Africa, as the

frequencies of transmission of different CMGs by *B. tabaci* populations from geographically distant locations in Africa were not significantly different (Maruthi *et. al.,* 2002).

Polyclonal antibodies originally raised by injecting a rabbit with purified particles of what was later described as the type isolate of ACMV from western Kenya have since detected geminivirus infection in CMD-affected cassava from more than 20 countries in Africa and the Indian subcontinent (Harrison *et. al.,*, 1995). The double-antibody sandwich version of enzyme-linked immunosorbent assay (DAS-ELISA), in which the antibodies are conjugated to alkaline phosphatase, has proved to be convenient and successful and is usually able to detect the viral antigen in extracts of young symptom-bearing cassava leaves at a sap dilution of 1:100, and in some instance 1:1000. However, mosaic-affected plants of many cultivars typically produce flushes of symptom-bearing leaves interspersed with symptomless leaves, in which the virus is not detected by DAS-ELISA. This finding, and other evidence, indicates that the virus is unevenly distributed in cassava shoots and emphasise the importance of selecting young symptom-bearing leaves for diagnostic tests (Fargette *et. al.,* 1987).

Geminivirus infection of cassava can also be detected by murine monoclonal antibodies (MAbs) raised against CMGs and used in triple-antibody sandwich ELISA (TAS-ELISA). As with DAS-ELISA, TAS-ELISA with one of these MAbs (SCR 20) has detected geminivirus infection in cassava from more than 20 countries. Moreover, TAS-ELISA is somewhat more sensitive than DAS-ELISA, especially when readings are made after overnight incubation with substrate. It also has the advantage that the background reaction given by extracts of CMG-free cassava leaves is negligible.

The successful purification of CMBs paved the way for development of antibody-based diagnoses of viruses associated with CMD. Polyclonal antibodies have been used for the detection

of ACMV in cassava leaf samples by the double antibody sandwich (DAS) method of enzyme-linked immunosorbent assay (ELISA) and immunosorbent electron microscopy. The availability of a panel of monoclonal antibodies gave impetus for rapid detection and discrimination of CMBs using triple antibody sandwich-ELISA. Although diagnosis of CMBs by ELISA is versatile and can be used for large-scale testing of field samples in diagnostic surveys, its major limitation lies in its inability to distinguish different CMBs in mixed virus infections. In addition, similarities in the coat protein epitopes of recombinant CMBs such as EACMV-UG and their parental makes it difficult for these viruses to be successfully differentiated by ELISA.

The advent of the polymerase chain reaction (PCR) technique has advanced molecular diagnosis of CMBs in several African countries including Cameroon, Nigeria, Tanzania, Kenya, South Africa, and so on.

These assays were developed using oligonucleotide primers specific to the DNAA component of CMBs in most cases. Amplified DNA fragments were then analyzed using restriction enzymes, used in hetero duplex mobility assays or sequenced for profiling CMBs. Recently, a multiplex PCR assay was developed for simultaneous detection of ACMV and EACMCV in CMD-affected cassava plants. The development of this assay has enhanced the capacity for rapid and reliable assessment of CMBs in epidemiological studies and crop improvement and phytosanitary programs in many sub-Saharan African countries. Nevertheless, ELISA is still a valuable and affordable diagnostic tool in countries where the capacity for molecular diagnostics is often lacking. (Alabi *et. al.,* 2011).

Detection is also possible by nucleic acid hybridization tests with probes derived from viral DNA. Probes for DNA-A of a group an isolate hybridize with isolates of all groups. Polymerase chain reaction (PCR) amplification of geminiviruses including CMGs is now possible and has

been used to study the strains occurring in Uganda and elsewhere (Deng *et. al.,* 1997;Zhou *et. al.,* 1997). The PCR technique is suitable for diagnosing large numbers of samples for the presence of CMGs using generic/degenerate primers for virus identification. This technique has an added advantage because the PCR product can be analyzed further by restriction site analysis or by cloning the product for sequencing (Henson J. M. and French R. (1993).

Polymerase chain reaction (PCR)-based applications in plant molecular biology and molecular diagnostics for plant pathogens require good quality DNA for reliable and reproducible results. Leaf tissue is often the choice for DNA extraction, but the use of other sources such as tubers, stems, or seeds, is not uncommon. The extraction of DNA from different tissue sources often requires different protocols. DNA was successfully used for the detection of fungal and viral pathogens and the genotyping of cassava by PCR. Alabi *et. al.,* (2008).

CHAPTER TWO

2.0 MATERIALS AND METHOD

2.1 COLLECTION OF SAMPLES

Sixty (60) samples were collected by from 6 different farms within Oye Ekiti and the easting, northing and elevation point was recorded using Geographical Positioning System (GPS). The choice and collection of sample were not biased and it was not due to symptoms showed by the cassava plant.

The cassava leaves were plucked without the petiole and the number three on the plant was plucked,2 leaves was collected on each of the cassava plants and 10 samples was collected in each farms .

2.2 PRESERVATION OF SAMPLE

Each sample was placed on a newspaper and labeled with a paper tape, the sample was then moved to the virology and molecular diagnostics lab in Germplasm health unit at International Institute of Tropical Agriculture (IITA), Ibadan.

TABLE 1: GEOGRAPHICAL POSITIONING STATION READINGS

SN	EASTING	NORTHING	ELEVATION	REMARK
01	31N 0755705	UTM 0860468	523m	FARM 1
02	31N 0755708	UTM 0860466	525m	FARM 1
03	31N 0755710	UTM 0860463	528m	FARM 1
04	31N 0755711	UTM 0860463	539m	FARM 1
05	31N 0755723	UTM 0860466	530m	FARM 1
06	31N 0755725	UTM 0860463	526m	FARM 1
07	31N 0755729	UTM 0860466	526m	FARM 1
08	31N 0755731	UTM 0860477	529m	FARM 1
09	31N 0755733	UTM 0860474	527m	FARM 1
10	31N 0755736	UTM 0860474	527m	FARM 1
11	31N 0758960	UTM 0864075	580m	FARM 2
12	31N 0758931	UTM 0864064	582m	FARM 2
13	31N 0758927	UTM 0864063	584m	FARM 2
14	31N 0758925	UTM 0864062	585m	FARM 2
15	31N 0758923	UTM 0864063	584m	FARM 2
16	31N 0758924	UTM 0864061	586m	FARM 2
17	31N 0758922	UTM 0864063	588m	FARM 2
18	31N 0758921	UTM 0864057	587m	FARM 2
19	31N 0758922	UTM 0864056	586m	FARM 2
20	31N 0758921	UTM 0864052	568m	FARM 2
21	31N 0757372	UTM 0862057	536m	FARM 3
22	31N 0757370	UTM 0862060	536m	FARM 3
23	31N 0757369	UTM 0862062	537m	FARM 3
24	31N 0757366	UTM 0862065	538m	FARM 3
25	31N 0757364	UTM 0862067	536m	FARM 3
26	31N 0757363	UTM 0862068	537m	FARM 3
27	31N 0757361	UTM 0862069	537m	FARM 3
28	31N 0757359	UTM 0862071	536m	FARM 3
29	31N 0757356	UTM 0862072	536m	FARM 3
30	31N 0757354	UTM 0862073	534m	FARM 3
31	31N 0755243	UTM 0862781	573m	FARM 4
32	31N 0755244	UTM 0862779	572m	FARM 4
33	31N 0755246	UTM 0862777	571m	FARM 4
34	31N 0755248	UTM 0862775	570m	FARM 4
35	31N 0755251	UTM 0862774	563m	FARM 4
36	31N 0755254	UTM 0862770	569m	FARM 4
37	31N 0755258	UTM 0862770	569m	FARM 4
38	31N 0755261	UTM 0862771	569m	FARM 4
39	31N 0755262	UTM 0862771	569m	FARM 4

SN	EASTING	NORTHING	ELEVATION	REMARK
40	31N 0755266	UTM 0862767	569m	FARM 4
41	31N 0755674	UTM 08161578	542m	FARM 5
42	31N 0755691	UTM 08161575	534m	FARM 5
43	31N 0755684	UTM 08161574	544m	FARM 5
44	31N 0755678	UTM 08161573	544m	FARM 5
45	31N 0755675	UTM 08161572	544m	FARM 5
46	31N 0755673	UTM 08161569	543m	FARM 5
47	31N 0755670	UTM 08161568	543m	FARM 5
48	31N 0755668	UTM 08161567	542m	FARM 5
49	31N 0755665	UTM 08161564	543m	FARM 5
50	31N 0755661	UTM 08161564	543m	FARM 5
51	31N 0755674	UTM 08613717	574m	FARM 6
52	31N 0755691	UTM 08613719	574m	FARM 6
53	31N 0755684	UTM 08613721	573m	FARM 6
54	31N 0755678	UTM 08613723	575m	FARM 6
55	31N 0755675	UTM 08613725	575m	FARM 6
56	31N 0755673	UTM 08613727	573m	FARM 6
57	31N 0755670	UTM 08613728	574m	FARM 6
58	31N 0755668	UTM 08613729	574m	FARM 6
59	31N 0755665	UTM 08613734	573m	FARM 6
60	31N 0755661	UTM 08613736	574m	FARM 6

2.3 NUCLEIC ACID EXTRACTION

DNA extraction was done by using CTAB/Dellaporta methods, 60 cassava leaves was placed inside a 60 mortar and pestle and 1ml of DNA extraction buffer which contains 60ml of CTAB buffer and 60ul of glycerol was added to the each mortal to aid grinding the cassava samples.

The cassava samples each was placed in an Eppendorf tube, was then vortex slightly. The vortexed Eppendorf tube is been arranged on a floated and then placed in a water bath at 60^0c for 10 minutes.

A mixture of phenol, chloroform and isoamyl alcohol was prepared for the samples in the ratio (25:24:1) respectively.600ul of the mixed reagent was then added to each of the heated samples.

The samples was the vortexed thoroughly. The samples was then spinned down using a centrifuge for 10mins at 12000 r.p.m.

The supernatant obtained was after centrifuge is transferred to a new eppendorf tube bearing same label as their parent, isopropanol was added to the sample.

The sample in the Eppendorf tube were shaken to allow the supernatant and isopropanol to mix together.

After mixing the whole sample were kept in the freezer at (-80^0c) for 1 hour.

After retrieving sample from the freezer they are the spinned for 10 minute at 12000 r.p.m a centrifuge, then decant the isopropanol

Isopropanol has separated from the DNA pellet formed by decantation.

To all the samples pellets 500ul of 70% ethanol was added in order to remove the trace of isopropanol left.

The sample are again spinned for 5minute at 12000 r.p.m, The ethanol 70% is then decanted out of the tube leaving behind the pellets.

The pellets in the Eppendorf tube is then dried in the incubator at 37^0c for 15 minute.

The dried pellet samples was then suspended with 50ul of distilled water.

2.4 THE INTEGRITY TEST FOR THE NUCLEIC ACID (DNA)

2.4.1 CASTING OF GEL

Three hundred (300ml) of Tris Acetic- EDTA was measured and 3g of agarose gel was weighed and microwave for 10 minute under moderate heated, and 15ul of dye to the microwaved agarose gel, then pour on a tray and leave for it to solidify.

The dye and the sample DNA was put inside each well 4ul of dyes was used and 4ul of each sample DNA was used, the DNA ladder (Promega) was put inside the first well, and each sample DNA and dye was (8ul) in each well, The power tanks was 110 voltage and was run for 30 minute.

2.5 PCR REACTION SET UP

Dilution method

The cassava DNA is first diluted inside in a ratio of (1:50).Each tube contain 2ul of template DNA (cassava samples) and 98ul of distilled water = 100ul solution.

Preparation of multiple PCR set up for cassava sample

Target virus ACMV, EACMV (oja mix) with sequences

TABLE 2: THE PRIMER, SEQUENCES, POSITION AND BASE PAIR

NAME	SEQUENCES	POSITION	BASE PAIR
ACMV AND EACMV oja rep-F	CRTCAATGACGTTGTACCA	Forward DNA	400 and 650
ACMV rep-R	CAGCGGMAGTAAGTCMGA	Reverse DNA	400
EACMV rep-R	GGTTTGCAGAGAACTACATC	Reverse DNA	650

DNA Reaction master mix X 65

The PCR total reaction volume was 12.5ul. This reaction contained

Sterile water; 6.69ul =434.85ul

PCR buffer (green); 2.5ul =162.5ul

dNTPs mM; 0.25ul =162.5ul

$mgcl_2$; mM 0.75ul =16.25ul

Oja mix; µmol 0.25ul =16.25ul

taq polymerase 0.3v;0.06ul =3.9ul

The sample was loaded inside a PCR tube using 10.5ul of the master and 2ul of each DNA sample comprising 2:50 diluted DNA, with correct labelling, sample 61,62,63,64 contain the control (EACMV and ACMV),while sample 65 contain the buffer

The reaction setup cycles are 1 cycle of 94°C [1 min], 52°C [2 min], 72°C [3 min]; 35 cycles of 94°C [1 min], 52°C [2 min], 72°C [1.33 min]; 1 cycle of 72°C [5 min]; 4°C [infinity]; End

2.6 RUNNING OF PCR PRODUCTS ON GEL

2.6.1 CASTING OF GEL

The gel was cast using 300ml of Tris Acetic- EDTA 4.5g of agarose was weighed and microwave for 5 minute, to dissolve the agarose completely, and 15ul of Ethidium bromide dye, then pour inside 200 and 100 tank of electrophoresis, allowed to solidify

2.7 LOADING OF PCR PRODUCTS

The gel is load with PCR product while lane 1 of each contain 5ul of the DNA marker.

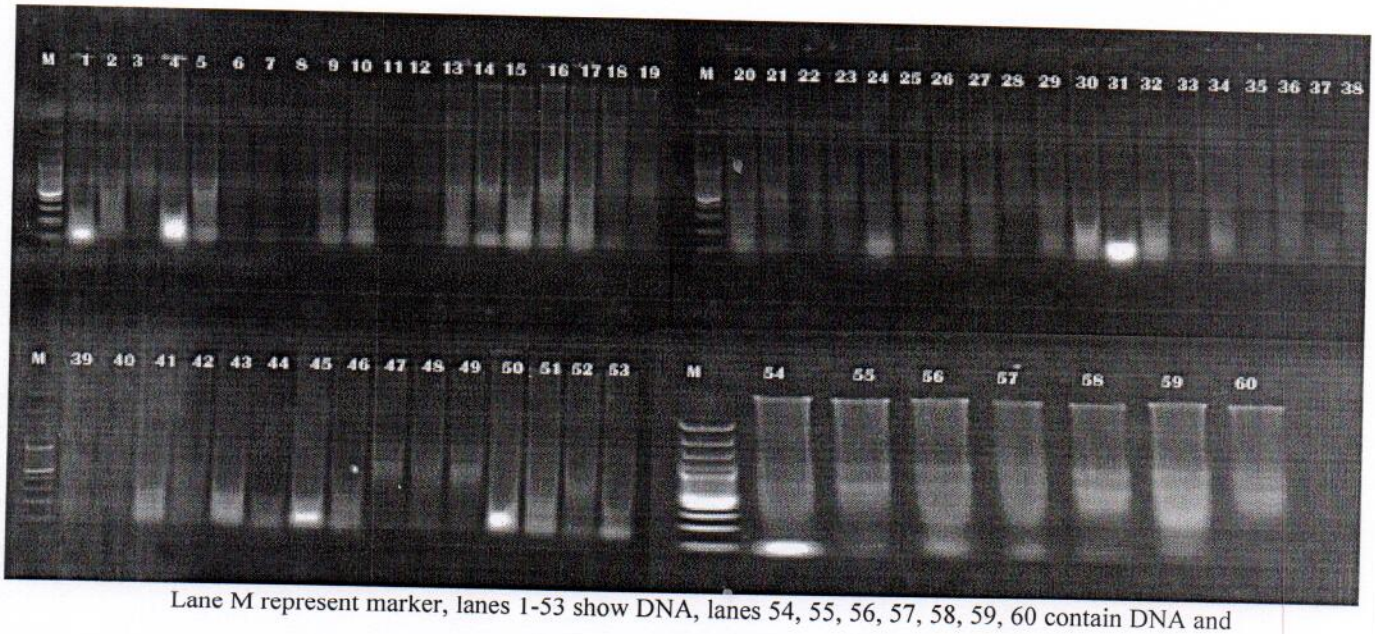
2.8 VISUALIZATION

Both the integrity gel and PCR product gel was view using a gel documentation (BIO RAD)

CHAPTER THREE

3.0 RESULTS

The integrity test done on DNA and RNA by gel electrophoresis on 1% agarose gel the samples show that the nucleic acid was of good quality and that extraction of nucleic acid using CTAB method was effective.



Lane M represent marker, lanes 1-53 show DNA, lanes 54, 55, 56, 57, 58, 59, 60 contain DNA and RNA.

Plate 3: A GEL VERIFICATION OF INTEGRITY TEST DONE ON THE NUCLEIC ACIDS EXTRACTED FROM CASSAVA LEAF SAMPLES FROM FARMS IN OYE EKITI, SOUTH WEST, NIGERIA

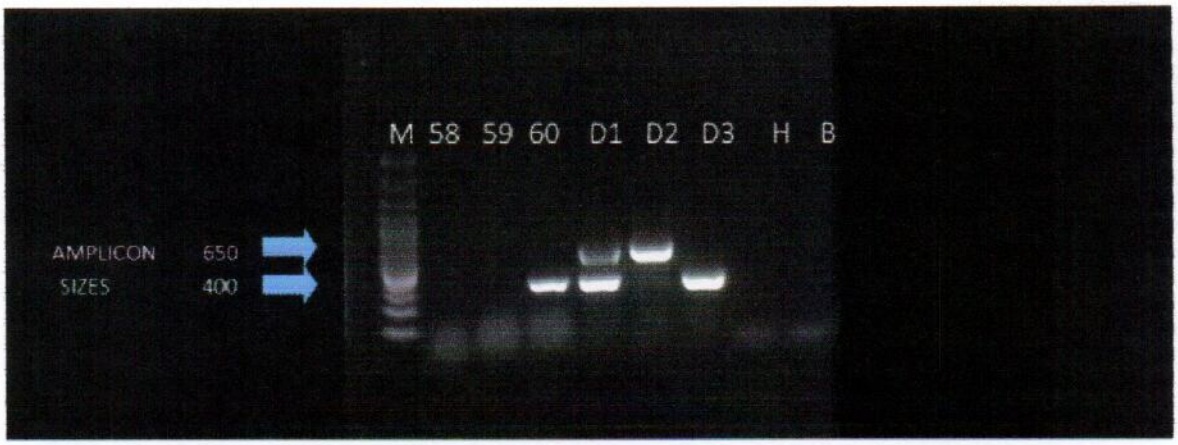
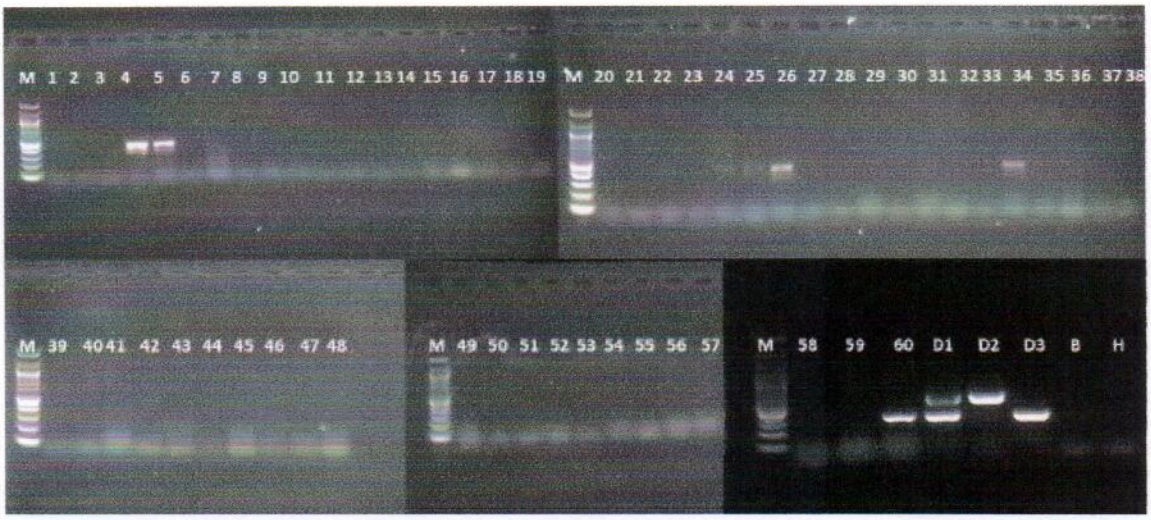


PLATE 4: A GEL VERIFICATION WITH ACMV AND EACMV, CONTROLS AND AMPLICON SIZES.

Electrophoresis Gel was on 1.5% agarose



Lane M represents lanes 4,5,24,25,26,34,60 show amplification with ACMV ,D1 show amplification with ACMV AND EACMV as a disease control,D2 show amplification with EACMV as diseased control,D3 show amplification with ACMV as a disease control B,H show no amplification with either of the virus as a healthy virus

PLATE 5: A GEL VERIFICATION OF PCR PRODUCTS FROM DNA OBTAINED FROM CASSAVALEAVES FROM OYE EKITI, SOUTH WEST, NIGERIA SHOWING AMPLIFICATION WITH ACMV, EACMV AND CONTROLS

TABLE 3: SUMMARY OF DISEASE STATUS FOR CASSAVA FARMS IN OYE EKITI TESTED FOR INFECTION WITH ACMV AND EACMV BY MULTIPLEX PCR USING PRIMERS FOR THE REPLICASE GENE

S/N	FARM	Infection status	ACMV	EACMV	Nucleic acid statu
1	FARM 1	-	-	-	D
2	FARM 1	-	-	-	D
3	FARM 1	-	-	-	D
4	FARM 1	+	++	-	D
5	FARM 1	+	++	-	D
6	FARM 1	-	-	-	D
7	FARM 1	-	-	-	D
8	FARM 1	-	-	-	D
9	FARM 1	-	-	-	D
10	FARM 1	-	-	-	D
11	FARM 2	-	-	-	D
12	FARM 2	-	-	-	D
13	FARM 2	-	-	-	D
14	FARM 2	-	-	-	D
15	FARM 2	-	-	-	D
16	FARM 2	-	-	-	D
17	FARM 2	-	-	-	D
18	FARM 2	-	-	-	D
19	FARM 2	-	-	-	D
20	FARM 2	-	-	-	D
21	FARM 3	-	-	-	D
22	FARM 3	-	-	-	D
23	FARM 3	-	-	-	D
24	FARM 3	+	+	-	D
25	FARM 3	+	+	-	D
26	FARM 3	+	+	-	D
27	FARM 3	-	-	-	D
28	FARM 3	-	-	-	D
29	FARM 3	-	-	-	D
30	FARM 3	-	-	-	D
31	FARM 4	-	-	-	D
32	FARM 4	-	-	-	D
33	FARM 4	-	-	-	D
34	FARM 4	+	+	-	D
35	FARM 4	-	-	-	D

S/N	FARM	Infection status	ACMV	EACMV	Nucleic acid statu
36	FARM 4	-	-	-	D
37	FARM 4	-	-	-	D
38	FARM 4	-	-	-	D
39	FARM 4	-	-	-	D
40	FARM 4	-	-	-	D
41	FARM 5	-	-	-	D
42	FARM 5	-	-	-	D
43	FARM 5	-	-	-	D
44	FARM 5	-	-	-	D
45	FARM 5	-	-	-	D
46	FARM 5	-	-	-	D
47	FARM 5	-	-	-	D
48	FARM 5	-	-	-	D
49	FARM 5	-	-	-	D
50	FARM 5	-	-	-	D
51	FARM 6	-	-	-	D
52	FARM 6	-	-	-	D
53	FARM 6	-	-	-	Dr
54	FARM 6	-	-	-	Dr
55	FARM 6	-	-	-	Dr
56	FARM 6	-	-	-	Dr
57	FARM 6	-	-	-	Dr
58	FARM 6	-	-	-	Dr
59	FARM 6	-	-	-	Dr
60	FARM 6	+	+	-	Dr
	Buffer		-	-	
	Healthy		-	-	
	Disease		++	++	

Key	
+++	Positive (+, ++, and +++ indicates strength of PCR amplification)
-	Negative
D	DNA only
R	RNA only
Dr	DNA and RNA

Overall Infection Status	
Total no of plants tested	60
Total infected	7
% infected	11.7

Total Accession

Total plants available for indexing	60
Total dead	0
% of Available plants	100
% of dead plants	0
Total %	100

ACMV

No of plants indexed	60
No of plants positive	7
No of plants negative	53
% positive	11.7
% Negative	88.3
Total %	100

EACMV

No of plants indexed	60
No of plants positive	0
No of plants negative	60
% positive	0
% Negative	100
Total %	100

CHAPTER FOUR

4.0 DISCUSSION

Integrity test carried out by Agarose electrophoresis of Deoxyribonucleic acid (DNA) extracts from 60 leaves using CTAB/ Dellaporta protocol, showed that they were of good quality. The samples were tested for ACMV and EACMCV in a multiplex polymerase chain reaction (PCR) with primers designed to amplify the **replicase** region of the DNA – a common feature of both viruses. The assay showed that most of the cassava plants within Oye Ekiti did not have the ACMV virus. The study also showed that the EACMV appeared not to be present in the area at all. Most positive samples were symptomatic. The low virus occurrence observed from PCR results and the observed low incidence of the CMD-characteristic mosaic symptoms on cassava leaves in the Oye Ekiti samples may be attributed to the use of CMD resistant or tolerant cassava varieties in the area.

.4.1 CONCLUSION

In conclusion, the multiplex PCR described above is suitable for rapid diagnostic studies requiring the specific detection and identification of CMDs in field-collected samples without the need for sequencing. Using the duplex and multiplex techniques, time was saved and the amount of reagents used was reduced, which translated into reduced cost of the diagnostic reagents.

The multiplex PCR in conjunction with a simplified sample preparation method is reliable, rapid, sensitive, specific and cost-effective for the diagnosis of CMDs in cassava plants. The assay is versatile since it can also be used for single virus detection using virus-specific primers. Consequently, this method is suitable for a wide variety of applications in many African countries for reliable assessment of the prevalence of CMDs in epidemiological studies and for crop improvement, quarantine, eradication and certification programs. The low incidence of the diseases shows the farmers in Ekiti area are using improved disease-resistant planting materials from nearby research institutes.

4.2 RECOMMENDATION

It is recommended that the multiplex PCR assay be used for rapid and extensive leaf sampling for cassava breeders screening for disease resistance, diagnostic studies, phytosanitary officers checking movement of diseased planting materials, and seed certification and multiplication officers for virus indexing. Farmers in other communities should be encouraged to also use certified planting materials for low disease incidence.

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