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DEVELOPMENT AND EVALUATION OF EFFICIENT DIAGNOSTIC TOOLS FOR CASSAVA MOSAIC AND CASSAVA BROWN STREAK DISEASES

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Dissertation submitted in partial fulfillment for the degree of Master of Science

The University of Witwatersrand, Johannesburg

Republic of South Africa

Declaration

This thesis is my work and has not been presented for a degree in any other University

Signed…….. Date: 28th August 2013

R.C. Aloyce

Dedication

This work is dedicated to my mother Fausta Manse and my late father Aloyce Kitona Mallya who unfortunately passed away before witnessing my MSc. Graduation. May God rest you in peace. Also, I dedicate this research to my young brother Cosmas Kitona for his support in the period of my studies.

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Research outputs

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List of acronyms

ABI	Applied Biosystems
ACMV	*African cassava mosaic virus*
ANOVA	Analysis of variance
MARI	Mikocheni Agricultural Research Institute
bp	Base pairs
BecA	Biosciences Eastern and Central Africa
°C	Degrees Celcius
CBSD	Cassava brown streak disease
CBSUV	Cassava brown streak Uganda virus
cDNA	Complimentary DNA
CMB	Cassava mosaic Begomovirus
CMD	Cassava mosaic disease
CP	Coat protection
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRC	Democratic Republic of the Congo
EACMV	*East African cassava mosaic virus*
EACMCV	*East African cassava mosaic Cameroon virus*
EACMKV	*East African cassava Kenya mosaic virus*
EACMMV	*East African cassava Malawi virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
EDTA	Ethylene-diaminetetraacetate

ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agricultural Organization
HCl	Hydrochloric acid xx
LSD	Least significant difference
M	Molar
masl	Metre above sea level
$MgCl_2$	Magnesium chloride
Ml	Millilitre
Ng	Nanogram
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribose nucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate and EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
UgV	Ugandan variant
μM	Micromolar

Abstract

Cassava (*Manihot esculenta* Crantz) is affected by two major viral diseases, namely Cassava brown streak disease (CBSD) and Cassava mosaic disease (CMD). Accurate and efficient detection and identification of plant viruses are fundamental aspects of virus diagnosis leading to sustainable disease management. In the present study I describe two techniques, the first based on a single tube duplex and multiplex polymerase chain reaction (m-PCR), developed for simultaneous detection of *African cassava mosaic virus* (ACMV), *East African cassava mosaic Cameroon virus* (EACMCV) and *East African cassava mosaic Malawi virus* (EACMMV), and second, a technique based on Restriction Fragment Length Polymorphism (RFLP) analysis of Reverse Transcribed (RT) -PCR amplified Cassava brown streak viruses species, *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV). In this work, the single tube duplex and multiplex PCR for simultaneous detection of the four cassava mosaic begomoviruses (CMBs) was developed successfully. Four primer pairs were designed from published DNA-A component sequences targeting specific amplification of the four cassava mosaic begomoviruses (CMBs). Evaluation of the primers sensitivity in serially diluted virus samples revealed that the new primers amplified their target virus to a dilution of 10^{-4} and 10^{-3} for uniplex and multiplex PCR respectively. Developed multiplex assay enabled specific amplification of the viruses in producing 950, 503, 435 and 260 base pairs (bp) for ACMV, EACMMV, EACMCV and EACMZV respectively in single and mixed infections of CBSVs. Analysis of 172 field samples from Kenya, Malawi, Mozambique, Rwanda, Tanzania and Zambia detected both single and mixed infections, results which were proved by analysis of the sequenced amplicons. Second, a technique based on

Restriction Fragment Length Polymorphism (RFLP) analysis of RT-PCR amplified cassava brown streak viruses, *Cassava brown streak virus* (CBSV) and *cassava brown streak Uganda virus* (CBSUV), was performed. A degenerate primer amplifying 785 bp of the coat protein gene (CP) of CBSV and CBSUV was also designed. Two restriction endonucleases, *Hind*III and *EcoR*1 (identified by a software package, Vector NTI® Express v1.0 from Life Technologies/Invitrogen), which produce different fragments upon digestion of RT-PCR amplicons from CBSV and CBSUV, were used to distinguish the two viruses RFLP analysis using *Eco*RI has no site in CBSV producing one fragment (785 bp), two fragments (525 bp and 224 bp) for CBSUV and three fragments (785, 525 and 224 bp) for the mixed infections. On the other hand, *Hind*III has no site in CBSUV producing one fragment (785 bp), three fragments (437 bp, 267 bp and 81 bp) were produced for CBSV, and four fragments (785, 437, 267 and 81 bp) for CBSV and CBSUV mixed infections. In both multiplex and RFLP analyses, results from the sequenced PCR/RT-PCR amplicons agreed with sequence identities of the respective published virus species. Experience from using developed multiplex and RFLP techniques show that time was saved and amount of reagents used were reduced. RFLPs confirmed the presence of CBSV and CBSUV in RT-PCR amplicons without requirement for sequencing. Additionally, modified protocols from Dellaporta *et al*. (1983) and Chang *et al*. (1993), were used to extract DNA and RNA respectively from dry and fresh cassava leaves with comparable results. I also demonstrated a method of collecting and preserving cassava leaf samples to retain their integrity during storage for a period of over one month. The two diagnostic tools can be used routinely in germplasm indexing, disease surveillance, and disease monitoring programs

Problem Statement and Rationale

In east and southern Africa, cassava (*Manihot esculenta* Crantz) is one of the leading crops in terms of production and has become an important source of income to households and small-scale farmers. However, the production across the region is greatly affected by Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD). Reports from different authors (Gibson. 1996; Ogbe *et al*., 1996; Legg *et al*., 1999; Fondong *et al*., 2000; Bisimwa *et al*., 2012) have reported the occurrence of CMD in different countries in the SSA. In Tanzania, CMD has been reported from many locations. Comprehensive characterization by Ndunguru *et al.* (2005) showed seven cassava mosaic geminiviruses species occur in Tanzania. Mbanzibwa *et al*. (2009a) reported prevalence of two potyvirus species causing CBSD in the Lake Victoria basin and along the coastal belt of Indian Ocean. A countrywide survey of all major cassava-growing areas in Kenya by Bull *et al.* (2006) reported presence of six CMG species with novel begomoviruses and a new recombinant strain of EACMV, demonstrating increasing diversity and geographical distribution of CMGs. Similarly, recent reemergence of CBSD has been reported in many districts in Uganda (Alicai *et al*., 2007) as well as from Malawi (Winter *et al*., 2010), Kenya (Mware *et al*., 2009) and Rwanda (Shirima *et al*., 2012). No reports of occurrence of CBSD have been reported from Zambia.

With the current development of more robust diagnostic tools such as RT-PCR and real-time PCR, the diagnosis of CMD and CBSD has also improved in many cassava-producing countries. Similarly, the challenges to obtain more sensitive broad-spectrum cost-effective diagnostic tools also increase. This is evident following discovery of more

virus species causing CMD and CBSD (Mbanzibwa *et al.*, 2009a and Winter *et al.*, 2010) which can easily be overlooked.

In the field the co-infections of many CMBs and CBSVs is common. Therefore, it will require several tools to detect the multiple infections using the diagnostic tools currently available. Thus, development of efficient and affordable diagnostic tools for simultaneous detection and identification of CMBs and CBSVs is vital and will have a significant impact on development and implementation of cassava virus disease management. Diagnostics will be used for disease monitoring in cassava multiplication plots production and distribution of disease- free cassava planting materials.

Therefore, this research make use of the available sequence information in the database for both CMBs and CBSVs to develop sensitive tools for the simultaneous detection of four species of cassava begomoviruses namely: *African cassava mosaic virus* (ACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV*) and East African cassava Mosaic Zanzibar Virus* (EACMZV) using multiplex PCR. Also identification and differentiation of two species of *Cassava brown streak viruses* namely *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) by RT-PCR/RFLP approach. This study generated knowledge and new tools that will enhance the diagnosis of both CMD and CBSD. The tools will facilitate deployment of virus-indexed cassava planting materials within the region.

Overall objective

This proposed study aims at enhancing the capacity of national cassava programs in the east and southern African countries to develop diagnostics tools to effectively implement disease management programs through control of CMD and CBSD.

The specific objectives of the study were;

i. To develop a sensitive and effective multiplex diagnostic tool for CMD and CBSD infecting virus species.
ii. To develop and optimize a reverse transcribed polymerase chain reaction/ restriction fragment length polymorphism tool for detection and differentiation of CBSVs species infecting cassava
iii. To evaluate the sensitivity of the tools in single and mixed infections in cassava

CHAPTER ONE

LITERATURE REVIEW

1.1 Cassava (*Manihot esclulenta* Crantz)

1.1.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a species native to tropical America (Olsen and Schaal 2001). The genus *Manihot* comprises 98 species spread throughout the Neotropics (Rogers and Appan, 1973). It has been cultivated in tropical America for more than 5,000 years. Cassava was first cultivated by native Latin Americans and then brought into Africa and Asia by the Portuguese traders in the 16th century (Jennings, 1976; Jones, 1959). According to FAO (2012) cassava market summary, quantity of cassava produced in Africa constitutes about 53% of the world production estimated at 230 million tons. The other major cassava producing continents (Figure 1) are Asia with approximately 30% from estimated 12 and 3.9 million ha of cassava cultivated land respectively (**Table 1**). Average yield of world cassava production in 2010 was estimated to be 12.40 tons/ha (FAO, 2012) (**Table 1).** In the region, cassava productivity differs remarkably between countries. Annual cassava productivity in Malawi, Mozambique, Kenya, Rwanda, Tanzania, Uganda and Zambia was 20.43, 6.0, 5.25, 12.04, 5.5, 12.75 and 5.82 tons/ha respectively (**Table 1**).

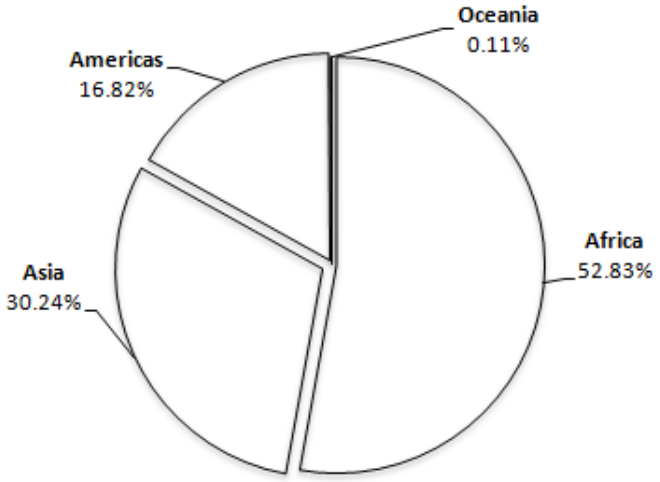


Figure 1: Cassava production share by world regions (FAO, 2012)

Table 1: Cassava productions in the world and in the region in 2010

Location	Area harvested (ha)	Yield (tons/ha)	Production (tons)
World	18,568,788	12.40	230,265,639
Africa	11,969,784	10.16	121,661,234
South America	2,400,720	13.20	31,686404
Asia	3,901,877	19.26	75,148,313
Tanzania	798,000	5.50	4,392,170
Kenya	61,573	5.25	323,389
Uganda	415,000	12.73	5,282,000
Malawi	195,828	20.43	4,000,990
Mozambique	950,000	6.00	5,700,000
Rwanda	197,394	12.04	2,377,210
Zambia	198,000	5.82	1,151,700

Source: FAO, 2012

1.1.2 Description of cassava

Cassava is a tropical perennial shrub that can grow to a height of 3m. It has erect smooth stems radiating from the roots. The stems contain nodes at intervals that give rise to new plants. Leaves are large lobed, borne on a long, slender stalk joining a leaf. The colour of leaves appear dark green but in some varieties yellow or purple pigmentation may occur

(Purseglove, 1968). Male and female flowers are found on the same plant. In some varieties of cassava cyanide producing sugar derivative occurs in varying amounts. There are many wild relatives of cassava. However based on morphological, ecological, and geographical evidence, Rogers, (1963) listed *M. carthaginensis, M. aesculifolia, M. grahami, M. flabellifolia,* and *M. saxicola* as the most closely related species to cultivated cassava.

Cassava is known to be drought tolerant crop. However, growth and yield of cassava is best in warm, humid tropical conditions. Generally, well distributed rains (ranging from 100 to 200 cm) during the growing months are considered ideal. Cassava is one of the most adopted crops in African Agriculture. It is cultivated in about 40 African countries. Wide spread adaptations of cassava in different soil and environmental conditions is caused primarily by the physiological traits possessed. El-Sharkawy, (2007) reported some of these characteristics to be: the high photosynthetic capacity of cassava important for high productivity; possession of a tight stomatal control over leaf gas exchange and ability to shade leaves, reducing reduces water losses during dry spell; ability to extract water from deep soils which enables plant to extract water in seasonally dry and semi arid environments and, ability to rapid multiply through cuttings. Additionally, Jarvis *et al*., (2012) pointed out that, cassava is a crop with high flexibility in adjusting to future climatic changes and therefore has a potential to become a crop of choice when other food crops are challenged.

1.1.3 Importance of cassava

Cassava has a reputation as a poor person's crop, i.e. a crop of last resort (Hillocks *et al*., 2001a). Generally cassava is used as food, industrial raw materials and substitute in animal feeds. About 90% of cassava root production is utilized as food and it is an important source of carbohydrates. In Africa cassava is produced mainly by smallscale farmers on marginal and sub-marginal lands. The bulk of cassava grown in Africa is utilized as food in the form of fresh roots and processed products such as flour and fermented meal preparations (Kawano, 2003). Furthermore, cassava leaves are consumed as vegetables and are the source of proteins and minerals (Lancaster and Brooks, 1983). Cassava leaves contain an average of 21% protein, which is high among non-leguminous plants (Ravindran, 1993). Cassava is transformed into a wide range of traditional product. The world production of cassava roots has reached 250.2 million metric tones of which trade accounts for about 10% of the total production (**Table 2**). Trade involves cassava for both human consumption and industrial use. Industrial use of cassava involves production of such commodities as ethanol, binding agent, paper, textiles and flavoring agent in Asian cooking and starch.

Table 2: Current world cassava root proportions 2010 to 2011 and forecast for 2011 (FAO, 2012)

Year	**2009**	**2010**	**2011 (forecast)**	**** (%)**
Total production	241.9*	237.9	250.2	5.2
Trade	25.6 (10.6%)	23.2 (9.8%)	22.8 (9.1%)	-1.8

*Million tons fresh roots
** Changes 2010 over 2011

1.1.4 Production constraints

Cassava production in sub-Saharan Africa is particularly exposed to numerous biotic stresses. Common constrains include pests and diseases, poor agronomic practices, high cyanide levels, lack of clean planting materials, low yielding varieties, and long maturity periods (Thresh *et al*. 1994). Pests and diseases are the most economically important constrains (Herren, 1994) to the cassava production. Pests infesting cassava include mealy bugs (*Phenacoccus manihot*), green spider mite (*Mononychellus tanajoa*) cassava green mite (*Mononychellus tanajoa*), cassava hornworm (*Erinnyis ello*), scales, thrips and whitely (*Bemisia tabaci) (*Montero, 2003*).*

Diseases among others include cassava bacterial blight, cassava virus diseases, cassava anthracnose disease, cassava bud necrosis, and root rots (Calvert and Thresh, 2002). Economic importance of cassava diseases depends on the extent of damage a disease causes to the productive part of cassava. In sub-Saharan Africa virus diseases of cassava are the most important (Taylor and Fauquet 1997; Thresh *et al*. 1994; Thresh *et al*. 1997). Cassava is reported to be vulnerable to at least 20 different viral diseases among which CMD and CBSD are the most devastating diseases (Patil and Fauquet, 2009). Sources of CMD and CBSD in cassava are believed to be viruses already present in the indigenous African flora (Legg and Hillocks, 2003). Factors influencing perpetuation of the virus diseases in cassava plant include: abundance of efficient insect vectors for transmission, planting of susceptible varieties and continuous use of unclean planting materials normally selected from the previous seasons.

With the evident success on biological control of cassava mealy bug and cassava green mite, CMD and CBSD remained the challenge. More information on the causative pathogens and efficient diagnostic tools are the prerequisite for the formulation of sustainable management approaches. Thus, CMD and CBSD are now one of research priority of many root and tuber crop programs in many African countries (Legg and Thresh, 2003).

1.2 Cassava Mosaic Begomoviruses

Cassava mosaic disease (CMD) is caused by cassava mosaic begomoviruses (CMBs), and was first described from what is now Tanzania towards the end of the 19th century (Warburg, 1894), and constitutes one of the most widespread and devastating diseases of cassava in Africa (Bock and Woods, 1983; Thresh *et al*., 1998). Early studies by Zimmerman, (1906) suggested that CMD is caused by a virus; however for many years viral etiology of CMBs remained unclear until 1938 when another study by Storey and colleagues from Aman research station in north eastern Tanzania confirmed that the disease is caused by cassava mosaic geminiviruses (CMGs) (family; *Geminiviridae*: genus; Begomovirus) (Storey, 1936). The virus is systemically transmitted in a persistent manner by whitefly (*Bemisia tabaci* Gennadius) (Homoptera: *Aleyrodidae*) (Dubern, 1994).

CMBs greatly reduce the growth and yield of cassava particularly local unimproved varieties (Thresh *et al*., 1997). CMBs spread easily from one field to another through planting of infected stem cuttings from the previous crop (Fauquet *et al*., 1988). Incidence, spread, severity and the extent of yield loss depend on the variety

susceptibility and stage of plant growth at which infection occurs. Recently, it was established that the severity of CMD is influenced by synergistic effects of co-infection of CMBs and its associated DNA satellites (Ndunguru *et al.*, 2008). Losses are attributed to damage on leaves and stems, which interfere with the way in which the plant makes food for storage in the roots. The damaged photosynthesis areas reduce the growth of the plants, number of storage roots and the ability of the storage roots to enlarge and mature. Loss of planting material also occurs in infected cassava, where stem cuttings are unhealthy and unsuitable for planting.

1.2.1 Cassava mosaic begomovirus structure

Viruses of the family *Geminiviridae* comprise a single–stranded DNA genome that is encapsidated in characteristic twinned (so called geminate) particles (Bull *et al*., 2006) The genome consist of two parts namely DNA-A and DNA-B components (Morris *et al*., 1990; Stanley *et al*., 2005; Bull *et al.*, 2006). DNA-A component replicates autonomously (Rogers and Appan, 1973; Klinkenberg and Stanley, 1990) and comprises of six specific protein encoding open reading frames (ORFs), AV1 & AV2 on the virion-sense strand, and AC1-AC4 on the complementary sense strand (Figure 2). AC1 encodes for replication associated protein (Rep) required for initiation of viral DNA replication, AC2 gene encodes for transcriptional activator protein (TrAP) that control gene expression, AC3 encodes for replication enhancer protein (REn) while RNA silencing suppressor protein is coded by AC4 gene. AV1 on virion-sense strand codes CP responsible for virus transmission from plant to plant by whitefly (*Bemisia tabaci)* and AV2 for pre coat protein (Patil and Fauquet, 2009). Replication of DNA-B depends on DNA-A. DNA B has two ORFs one each on the virion and complementary strand; BV1 is a shuttle protein

encoder (NSP) while BC1is responsible for movement protein (MP) encoding. The virus move within and between cells of host plants by a co-operative action of the two genes (Hanley-Bowdoin *et al*., 2004). Virus infection and subsequent symptom development in host plant requires presence of both virus components (DNA-A and DNA-B) (Stanley and Gay, 1983).

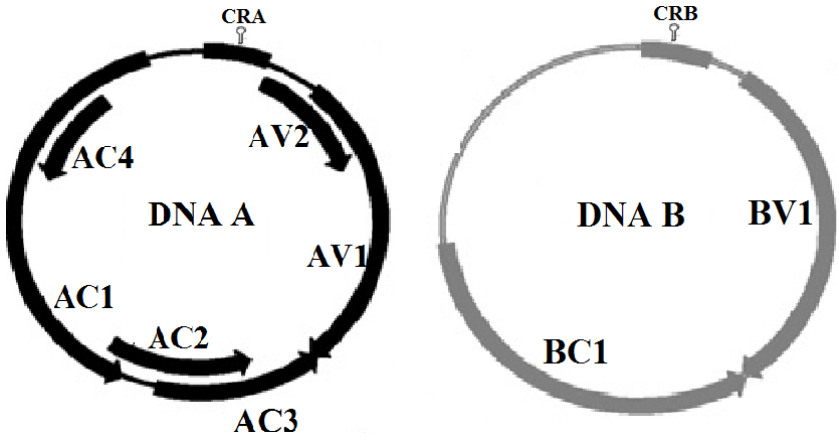


Figure 2: Cassava mosaic begomovirus genome organization composed of twinned particles; DNA-A (left) and DNA-B (right). Functional ORFs on the DNA-A virion-sense (AV1 & AV2) and the complementary-sense strand (C1 to C4). DNA-B has two ORFs: BV1 and BC1. In both DNA-A and DNA-B component there is a non-coding intergenic region referred to as the common region (CR). (Adapted from Patil *et al*., 2007).

1.2.2 Cassava mosaic begomovirus species

Taxonomic guidelines developed recently (Fauquet *et al*., 2008) provided a frame work for defining species and strains. This approach sets sequence demarcation between members of different species to be 89% of begomoviruses DNA-A component nucleotide sequences. Using this criterion seven distinct but similar virus species of CMD in Africa and 2 in Indian sub-continent have been described. These are; *African cassava mosaic*

virus (ACMV*), East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV) and *South African cassava mosaic virus* (SACMV). India subcontinent has Indian *cassava mosaic virus* (ICMV) and *Sri Lanka cassava mosaic virus* (SLCMV). Each of these species can induce CMD on cassava plants both in single and in co-infections resulting in severe disease (Harrison *et al.*, 1995; Thresh *et al*., 1998; Berry and Rey, 2001).

1.2.3 Geographical distribution

Earlier understanding on the distribution of the CMD viruses was that EACMV and ACMV occupied distinct but largely non-overlapping geographical areas, were ACMV occurred West, Central and Central Southern Africa, while EACMV was restricted to the East African coast, Madagascar, Malawi, Mozambique and Zimbabwe (Harrison *et al*., 1991, 1995). Subsequent studies (Gibson. 1996; Ogbe *et al*., 1996; Legg *et al*., 1999; Fondong *et al*., 2000; Bisimwa *et al*., 2012) showed that EACMVs occurs in a much wider area including Western Kenya, Western Tanzania, Zambia, Nigeria, Togo, Guinea, Ivory Coast, Cameroon and Democratic Republic of Congo. Distribution map of cassava begomoviruses based on ELISA- and PCR- results of samples collected between 1998 and 2001 from Africa shows that ACMV was detected in all cassava-growing countries in the continent a region ranging from the savannah zones of the Sahel to the northern regions of South (Were *et al*., 2003). Earlier report showed EACMV was restricted to the coast of East Africa while ACMV occurred in all cassava growing areas of the continent (Swanson and Harrison, 1994). Currently, however it has been established that EACMV

has extended its occurrence away from the coastal regions. It has now been reported from Western Kenya, Western Tanzania, North-Eastern Zambia, Nigeria, Togo, Cameroon and Uganda (Ogbe *et al*., 1996; Legg and Okao-Okuja, 1999; Fondong *et al*., 2000; Pita *et al*., 2001).

1.2.4 Cassava mosaic disease symptoms

Infected cassava plants exhibit a range of symptoms variation. Gibson and Otim-Nape, (1997) reported factors contributing to the variation in symptoms to include: types of virus strain, age of plant, host plant sensitivity and environmental such as moisture availability in the soil, fertility of the soil, solar radiation and temperature. However, characteristic symptoms of CMD infected plants are infected leaves show green to yellow mosaic, setting up unequal expansion in affected areas causing twisting, narrowing and malformation of the leaf. In condition of severe infections young leaves abscise and affected plant appear stunted and produce small fewer tubers These morphological alterations in cassava plants result in significant losses in storage root yield (Storey and Nichols, 1938; Seif, 1982; CABI., 2004).

1.3 Cassava Brown Streak Disease (CBSD)

Cassava brown streak disease (CBSD) was first described in the Amani district in the Tanganyika territory (now Tanzania) in 1930 (Storey, 1936). CBSD was reported to be a major threat to food security in the coastal regions of Tanzania (Legg and Raya1998; Hillocks *et al*., 1996), Northern Mozambique (Hillocks *et al*., 2002) and in the coastal strip of Lake Malawi. CBSD is caused by *Cassava Brown Streak Viruses* in the genus *Ipomovirus* in the family *Potyviridae* (Monger *et al*., 2001a).

Economic losses are due to damage inflicted in the above ground plant parts and also spoilage of below ground roots due to dry necrotic rot. Hillocks *et al*., (2001b) reported a yield loss of up to 100% in when susceptible cassava was grown in high disease incidence areas in Tanzania. CBSD has also significant effect on cassava tuber weight loss due to root necrosis, which makes a large portion of tubers unfit for both human and animal use.

1.3.1 Genome structure and organization of CBSV

The genome of CBSV is about 9kb composed of positive sense single stranded linear ssRNA, and a poly (A) tail at the 3´end (Mbanzibwa et al., 2009b). Unlike members of type specie of Genus *Ipomovirus* (Collinet *et al.*, 1998) CBSV genome lacks helper component proteinase but contain PI serine proteinase that strongly suppressed RNA silencing (Mbanzibwa *et al*., 2009b). CBSV genome contains a single ORF, with UTR at the 5´ and 3´ends. It encodes a large polyprotein ca. (2902 aa) that is processed by virus-encoded proteases into mature proteins namely P1 proteinase (P1-Pro), the third protein (P3), 6kDa protein 1 (6K1), cylindrical inclusion protein that is an RNA helicase (CI), 6kDa protein 2 (6K2), nuclear inclusion protein a (NIa), which can be further processed into the viral protein genome-linked (VPg) and NIa proteinase (Pro). Beside exceptional structure of the 5'-proximal part of the genome CBSV also contained a Maf/HAM1-like sequence recombined between the replicase (Nib) and the coat proein domains in the 3'proxial part of the genome highly conserved in *Potyviridae* (Mbanzibwa *et al*., 2009b).

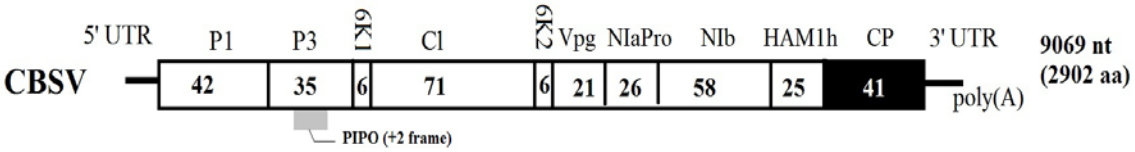


Figure 3: A schematic presentation of the genome structure of CBSV with a large ORFs that is translated into viral polyprotein indicated in the individual boxes. The estimated molecular weights of the mature proteins (in kilodaltons) are also indicated in the box

1.3.2 Geographical distribution

Early reports shows that CBSD was found only at altitude below 1000 meters above sea level (masl), predominantly in East African coast of Indian ocean (in Tanzania, Kenya and Mozambique) and in the lake shore areas of Malawi making it to be termed as a disease of cassava in lowland areas (Storey, 1936; Monger *et al*., 2001a, b; Hillocks and Jenning, 2003). Recent reports shows that CSBD is more wide spread and found at areas with higher altitude than 1000 masl as previously thought. These areas include Uganda (Alicai *et al*., 2007), Lake Victoria zone of Tanzania and Western Kenya (Mbazibwa *et al*., 2009a, Winter *et al*., 2010; Monger *et al*., 2010), Rwanda and Burundi (Shirima *et al*., 2012) and Democratic Republic of Congo (Mulimbi *et al*., 2012; Shirima *et al*., 2012).

1.3.3 CBSD etiology

Initial belief was that, CBSD was caused by a virus although no reliable information was available to confirm this claim. The first confirmation of viral cause came from Lister, (1959) sap inoculation experiment where he was able to transmit the disease by sap inoculation from cassava to herbaceous hosts and back transmission to cassava. By using electron microscopy Kitajima and Costa, (1964) observed elongated virus like particle in CBDS infected plant. Lennon *et al*., (1986) estimated the size of the virus particles to be

650-690 nm. CBSD was reported to be associated with two separate viruses, a carlavirus and a potyvirus (Brunt *et al*., 1990). Carlavirus cause was ruled out by the existence of pinwheel inclusions in cells of the diseased plants indicating a potyvirus presence (Harrison *et al*., 1995; Were *et al*., 2004). Evidence that CBSD is caused by Cassava brown streak virus belonging to the genus Ipomovirus and family *Potyviridae* was given by Monger *et al*., (2001b) from partial sequence information from the coat protein encoding sequences.

From analysis of CBSV isolates from infected cassava obtained from East Africa, Winter *et al*., (2010) had described two distinct virus species. Description was based on differences observed both in biological behavior and in genomic and protein sequences. He described CBSVs to be clustered in two distinct clades. Clade 1 was comprised of isolates from Malawi, Kenya, Uganda, North western Tanzania and the CBSV described previously, sharing between 87 and 95% nucleotide sequence identity while clade 2, was comprised of isolates from coastal areas of Mozambique and Tanzania which shared only 70% nucleotide sequence identities with isolates of the first clade. Virus in clade1 and 2 were called Cassava brown streak virus and Cassava brown streak Mozambique virus respectively.

The novel species, Cassava brown streak Mozambique virus and Cassava brown streak virus, were re-named as *Cassava brown streak virus* (CBSV) and C*assava brown streak Uganda virus* (CBSUV) by the International Committee for Taxonomy of Viruses in June, 2010. More understanding of CBSV and CBSUV has been brought to light by Mbanzibwa *et al*. (2009a, 2009b, 2011); Monger *et al*. (2010a, b) and Winter *et al*. 2010) from information obtained by sequencing partial and full genomes of the viruses.

1.3.4 Transmission spread and host range

The primary source of CBSV transmission to a new cassava plant is through to be the use of diseased planting materials. Storey (1936) demonstrated that characteristic foliar symptoms of the disease were obtained from cuttings from affected plants. Insect transmission of CBSV has also been suggested and that the most likely vector is the whitefly *Bemicia tabaci* (Gennadius) (Hemiptera: *Aleyrodidae*) (Storey 1939; Bock, 1994). Unsuccessful transmission attempt was done by Lennon *et al*., (1986); Brunt *et al*., (1990) and Bock *et al*., (1994). First whitefly transmission confirmation came from Maruthi *et al.,* (2005) whereby the whitefly *Bemicia tabaci* transmitted CBSV at a very low rate (maximum of 22%). Contrarily recent study by Mware *et al*., 2009 showed that CBSV is transmitted by *Bemisia tabaci* and spiraling whitefly (*Aleurodicus dispersus)* Russell (Hom, *Aleyrodidae*) with transmission efficiencies of 40.7% and 25.9% respectively. Other means of transmission of the CBSVs includes grafting, cutting tools and leaf harvesting. Experimentally, CBSV can be transmitted to *Nicotiana benthamiana* and *N. rustica*. So far cassava brown streak disease is not known to attack other crops and no known host range for CBSV reported to date. However, it is believed that CBSV must have an indigenous host from which it spread to cassava after being introduced to Africa (Calvert and Thresh, 2002).

1.3.5 CBSD symptoms

1.3.5.1 Above ground symptoms

Nichols (1950) elaborated two types of leaf symptoms: Secondary and tertiary veins yellow chlorosis and the secondly general blotchy chlorotic mottle. Both symptoms

appear on the lower mature leaves and vary from variety to variety. Symptoms may differ with varieties and do not appear on newly formed foliage especially at high temperatures. Symptoms can be also transient when a period of active growth produces symptom-free tissues (Jennings, 1960). Unlike CMD, CBSD do not induce leaf distortion or size reduction.

1.3.5.2 Stem symptoms

CBSV also induce brown necrotic streaks on the green portions of stems of cassava plants. The upper portion of the stem become necrotic and then dry out causing shoot dieback.

1.3.5.3 Below ground symptoms

The most economical importance of CBSD is the destruction of storage roots. A study by Hillocks *et al*. (1996) in Tanzania observed some cultivars do not show root necrosis until more than 8 months after planting using infected cutting despite the earlier presence of apparent foliar symptoms. Characteristic symptoms begin with small yellowish/brown, corky specks that increase in size and number until the whole root becomes inedible. Symptoms in roots become more intense as the crop matures particularly beyond physiological maturity at about 12 months post planting (Nichols 1950). Symptomatic roots also suffer from secondary infection caused by soil-borne pathogens and normally soft rot sets in.

1.4 Plant Disease Diagnostics: Status to date for CMBs and CBSVs

Plant disease diagnostics is a key step in diseases management providing a better understanding of the disease causative agents and the most appropriate way to their solutions. Plant pathologists have gained access to varieties of different diagnostic techniques (Fox, 1993). The form of diagnostic technique for a certain disease should be quickest, easiest, affordable and most accurate method, which can confirm the correct identification of the causal agent (Fox, 1990). There is a growing demand from producers for rapid and accurate diagnosis of pathogens to guide disease management decision making and issue of phytosanitary certificates (McCartney, *et al*., 2003; Miller, 1995). There are several methods (diagnostic tools) available nowadays but for a diagnostician and epidemiologist one has to know the limitation of each method and choose the most appropriate. Due to the fact that some plant pathogens evolve into different strains in

different ecological/geographical locations reliability to a diagnostic tool developed in one location has been inadequate when used in another location (Garcia *et al*. 2003; Garcia and Fraile, 2008; Sacristan and Garcia, **2008**). It is necessary to modify existing diagnostic tools or develop new techniques suitable to pathogen strains of a given locality. The quality of plant disease diagnostic depends on the availability and quality of human capital, infrastructure, and technology (Miller *et al*., 2009). Diagnostic procedures for both CMD and CBSD viruses in East and Southern Africa are done using a combination of methods that have been developed to effectively identify different species of the viruses occurring in the region depending on level of expertise available and capacity of most laboratories in the region.

1.5 Diagnostic tools currently used for cassava virus diseases

1.5.1 Methods of detection based on biological properties

1.5.1.1 Symptomatology

Symptoms recognition has always been used by scientists as the first step towards identification of both CMD and CBSD in cassava plant. It is used to establish disease incidence and severity. It starts with visual inspection for characteristic symptoms of a specific disease. However, there is always a challenge as noted by Mathews, (1980) that many factors influence virus symptom expression including plant variety/cultivar, virus strain, stage at which a plant is infected, and the environment. Also when plant is subjected to unfavorable growth conditions can exhibit symptoms like those caused by the virus (van der Want *et al.*, 1975). In some cases virus do not induce noticeable symptoms or infect a host plants without causing any symptoms. Furthermore, a group of

different viruses can exhibit similar symptoms or same symptoms can be caused by different strains of a virus.

Diagnosis of plant virus disease based on expressed symptoms alone can provide a usefully information only when done by a person with a very good field experience. To strengthen further the reliability of visual inspection, information obtained by visual inspection is supplemented with other confirmatory tests to make sure that a correct disease has been diagnosed (Bock, 1983). The other tests can be microscopic techniques, isolation or both. If the putative virus that is isolated is unfamiliar, then its pathogenecity should be checked by satisfying Koch's postulate by re-inoculating the isolate into the host plant to produce the same symptoms (Fox and Narra, 2005). Generally, this method is time consuming and encircled with many problems. It needs substantial expertise and experience of a wide array of specialized technologies rendering it less attractive for routine use.

1.5.1.2 Pathogenicity or transmission tests

Pathogencity or transmission tests are usually done using indicator plants. Indicator plants are plants from some genera such as *Nicotiana* (tobacco) and *Chenopodium* (lambs quarters), which can host a number of viruses. Under green house conditions these plants usually react to viral infections in a distinct and consistent way and therefore used as indicator plants (Walkey, 1991). According to Jones, (1993) introduction of the virus into the indicator plant can be mechanically by grafts or vector transmission. Mechanical transmission through sap inoculation of CBSV onto *Nicotiana rustica, N. benthamiana, N. occidentale and N. tobacco* give rise to necrotic lesions (Bock 1994; Mbanzibwa *et al*., 2009a). Though systemic infection of *Nicotiana spp* does not give discernible symptoms,

CBSV can be detected from upper leaves using RT-PCR. Viruses of both CMD and CBSD have been detected through inoculation on to indicator plants. Although symptoms expression in indicator plants is still being used in many laboratories for maintaining virus cultures for diagnosis it is time consuming and expensive. It is also faced with same difficulties in viruses identification as in symptom expressed in the field.

Currently mechanical transmission using sap/graft transmission from infected onto health plant has enabled transmission of CBSD into a health plant. However, despite low transmission rate of CBSD by whitefly (Maruthi *et al.,* 2005) from diseased onto health plant, both white fly *Bemisia tabacii* and *Bemisia afer* are known to transmit CBSD. Mware *et al*. (2009) reported that acquisition feeding of *B. tabaci* for 48 hours on CBSD-infected cassava leaves, CBSV transmission rate was 40.7% compared to 25.9% of spiraling whitefly (*Aleurodicus disperse).*

1.5.2 Immunology

An immunological test is based on identification of target viruses through antigen-antibody specific interaction. Tested virus is the antigen, which reacts with specific antibodies. Specific antibody for a particular plant virus is made by injecting a purified plant virus and allowing the animal body to react to the injected virus for several weeks and them the animal blood serum is collected and antibody separated from the blood. The resulting antibody will react specifically to the viral protein injected into the animal (Fox, 1993). Several different serological assays that utilize solid phase support exist (Bar-Joseph *et al*., 1979; Rocha-Pena and Lee, 1991). These include dot immunoblotting assay, western blotting, radio-immunoassay, immune electron microscopy, immuno-

fluorescence microscopy; immuno-gold EM, but the most commonly used is the enzyme linked immunosorbent assay (ELISA). Taking advantage of the specific interaction between antibody and antigen, screening kit for plant viruses have been develop and serological methods are currently being used for rapid detection of plant viruses from different crops (Torrance, 1998; Prasangika *et al*., 2008).

In cassava virus diseases, ELISA with monoclonal antibodies (Mabs) was developed to distinguish two viruses currently known as ACMV and EACMV (Swanson and Harrison 1994; Harrison *et al*., 1997). ELISA kits have been developed and are commercially available (Ogbe *et al*., 1996; 1997). With recent emergency of viral sequence information from CBSVs, serology-based ELISA kits has been developed for detection of several species and/serotypes of CBSVs (Winter *et al.*, 2010). ELISA assays are cheap and quick method for routine works but its sensitivity requires fresh leaves with clear symptoms. These requirements limit ELISA ability to detect viruses at early stage of infections or in plants having latent symptoms. In a condition where more than one virus species are infecting the same plant the non-targeted viruses will pass undetected. Thus, identification of the virus/strain in question depends on addition of a more sensitive tool such as PCR and sequencing. The more sensitive methods employed, the greater the probability the diagnosis will be correct.

1.5.3 Molecular hybridization techniques

Molecular hybridization technique works on the principle of specific pairing of the bases composing nucleic acids to form hybrids (double-stranded structure) between complementary molecules. The technique requires a DNA probe, which is usually a

fragment of labelled gene whose sequences are complimentary to the tested virus sequence.

There are different forms of molecular hybridization techniques: i) Southern blotting; a technique devised by Southern in 1975 to detect sequences in a DNA mixture. It involves transfer of DNA molecules from an electrophoresis gel onto a nitrocellulose or nylon membrane. ii) Northern blotting technique is a technique similar to southern blotting used to detect sequences in an RNA mixture and iii) Dot and slot blots both represent a modified northern and southern blotting. They involve direct application of the sample (target viral nucleic acid) onto the nylon or nitrocellulose membranes. The applied samples bound to a membrane by baking the membrane and followed by hybridization with a labelled probe. The detection of the target virus is achieved using autoradiography (for radioactive probes), or by a colorimetric reaction if an enzyme label is used (Meinkoth and Wahl, 1984; Sela *et al*., 1984; Pallas *et al*., 1998).

Hybridization has been used in plant virology first to detect viroids and later plant viruses (Maule *et al.,* 1983; Garger *et al.,* 1983). In cassava virus, dot-blot hybridization with non-radioactive probe was used to detect begomoviruses such as *South African cassava mosaic virus* (Berrie *et al*., 2001) and *African cassava mosaic virus* (Ettesami et al., 1991).

1.5.4 Nucleic acid based tools

1.5.4.1 Polymerase chain reaction (PCR)

PCR is an *in vitro* method in which a DNA fragment with known end sequences can be amplified exponentially into billions of copies, making detection very much easier (Saiki *et al.,* 1985). PCR is preferred in many areas of research due to its versatility, sensitivity and specificity. PCR has ability of amplifying target nucleotide even when it is present in a reaction mix at extremely low quantities and as a result it has been identified as a preferred technique for plant virus diagnosis (Henson and French, 1993; Candresse *et al*., 1998). Technology advances in PCR has resulted into invention of Real time PCR (qPCR). Contrary to the conventional PCR where detection of the target nucleotide is at the end-point of the reaction, qPCR enables detection while the reaction is occurring. The qPCR combines DNA amplification and detection in a single PCR reaction tube through a system of fluorescent reporter dyes. Results are presented as Ct-values. qPCR has higher sensitivity, more specificity, and provide scope for automation (Schmittgen and Livak, 2008). Although PCR/RT-PCR is extremely sensitive, it has the disadvantage that it is expensive, liable to contamination, not easy to set up quantitative assay and requires high degree of operator skills and therefore not available to many laboratories in Africa (Fauquet *et al.,* 2003; Ogbe *et al*., 2003; Fondong *et al.,* 2000).

1.5.4.2 Multiplex PCR (m-PCR)

In many cases plants are infected by a wide range of viruses that often cause complex single diseases. Molecular techniques have revolutionized the way of plant virus detection and identification by the use of multiplex PCR (RT-PCR). In multiplex

PCR/RT–PCR more than one oligonucleotide primers specific to certain viral species/strains are used in a single reaction tube to allow their identification simultaneously. Multiplex primers are designed to minimize cross reactivity and produce amplicons of different lengths for identification in agarose gel electrophoresis. Procedures that allow simultaneous detection/or identification of different viruses are desirable for routine diagnoses because they require less time, labour and cost (Kumar *et al*., 2009; James *et al*. 2006; Park *et al*., 2005). In this sense the multiplex PCR /RT-PCR has been used successfully for routine diagnosis of plant viruses and viroids (James *et al*., 2006). In cassava viruses multiplex PCR assays have being developed and used for detection of both CMBs and CBSV species. For example Alabi *et al*. (2008) multiplex PCR detected ACMV and EACMV, Kumar *et al*. (2009) simultaneously detected CBSV, ACMV and EACMV, Abarshi *et al*. (2012) detected CBSV species in combination with some of CMB species. Alongside the superior advantages of multiplex PCR (rt-PCR), this method shares the same draw back as in PCR. This method can be automated to target more viral targets using real time PCR and produce qualitative result in a very short

1.5.4.3 Restriction Fragment length Polymorphism (RFLP)

Restriction fragment length polymorphism is a molecular biological technique used to identify oligonucleotides based on fragment patterns produced by restriction endonucleases. Restriction endonucleases are enzymes that cleave DNA at specific recognition nucleotide sequences known as restriction sites (Roberts, 1976; Pingoud *et al*., 1993). RFLP can be used to cut a genomic DNA, plasmid or PCR amplicons generated by primers. The template to be cut is digested with appropriate restriction endonuclease under favorable temperature of the enzyme to produce restriction fragment,

which are then analyzed in agarose gel electrophoresis to produce identity pattern of the virus/nucleotide. RFLP has been used to identify virus species/isolates especially when sequence information is not relevant. Expenses of cloning and sequences are avoided in this method. For example RFLP has been used to provide distinction between ACMV and EACMV (Briddon *et al*. 1993; Sseruwagi *et al*., 2004; Ndunguru *et al.,* 2005). While there is well-documented information on RFLP assays for CMBs the same information is lacking for *CBSVs.*

CHAPTER TWO

A SINGLE-TUBE DUPLEX AND MULTIPLEX PCR FOR SIMULTANEOUS DETECTION OF FOUR CASSAVA MOSAIC BEGOMOVIRUS SPECIES IN CASSAVA PLANTS

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2.1. Abstract

A single-tube duplex and multiplex PCR was developed for the simultaneous detection of *African cassava mosaic virus* (ACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV) and *East African cassava mosaic Zanzibar virus* (EACMZV), four cassava mosaic begomoviruses (CMBs) affecting cassava in sub-Saharan Africa. Co-occurrence of the CMBs in cassava synergistically enhances disease symptoms and complicates their detection and diagnostics. Four primer pairs were designed to target DNA-A component sequences of cassava begomoviruses in a single tube PCR amplification using DNA extracted from dry-stored cassava leaves. Duplex and multiplex PCR enabled the simultaneous detection and differentiation of the four CMBs, namely ACMV (940 bp), EACMCV (435 bp), EACMMV (504 bp) and EACMZV (260 bp) in single and mixed infections, and the results corroborate sequence identities of the respective published virus species. In addition, I report here a modified Dellaporta *et al*. (1983) protocol, which was used to extract DNA from dry and fresh cassava leaves with comparable results. Using the duplex and 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 certification and multipliers for virus indexing.

Key words: Cassava, begomoviruses, detection, duplex, multiplex PCR

2.2. Introduction

Cassava mosaic disease (CMD) is the most limiting biotic factor to cassava (*Manihot esculenta* Crantz) production in sub-Saharan Africa (SSA) (Thresh *et al*., 1994). Yield losses of 20 to 95% have been reported in farmers' fields due to CMD (Fargette *et al*., 1988). The disease is caused by viruses belonging to the genus *Begomovirus,* family *Geminiviridae,* which are transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Dubern, 1994) and spread through planting of infected cassava stakes.

Nine cassava mosaic begomovirus (CMB) species have so far been reported to infect cassava worldwide (Fauquet *et al*., 2008). In SSA alone, seven of the cassava-infecting CMBs, namely *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), *East African cassava mosaic Kenya virus* (EACMKV) and *South African cassava mosaic virus* (SACMV) were reported (Fauquet *et al*., 2008).

Early efforts to detect the causative agents of CMD in SSA first employed Enzyme-linked immunosorbant assays (ELISA) with monoclonal antibodies (Mabs) to distinguish two viruses currently known as ACMV and EACMV (Swanson and Harrison 1994; Harrison *et al*., 1997). A key advantage of ELISA is that it is relatively simple to perform and requires fewer resources. However, ELISA requires fresh samples with clear disease symptoms, owing to its failure to detect viruses at very low titre. In addition, the tool is less sensitive and is unable to distinguish other CMBs such as EACMCV, EACMKV,

EACMMV, SACMV, EACMZV and the variant *East African cassava mosaic virus-Uganda* (EACMV-Ug), which has similar epitope profiles in the coat protein as ACMV.

A more definitive and sensitive tool was later developed called polymerase chain reaction (PCR), which targets the virus nucleic acids. Degenerate and virus specific primers were developed and subsequently used to detect the CMBs occurring in several African countries, including Uganda (Zhou *et al*., 1997; Fondong *et al*., 2000; Pita *et al*., 2001), South Africa (Berry and Rey 2001), Rwanda (Legg *et al*., 2001; Sseruwagi *et al*., 2005), Senegal (Okao-Okuja *et al*., 2004), Kenya (Were *et al*., 2004), Tanzania (Ndunguru *et al*., 2005) and Nigeria (Ogbe *et al*., 2006). However, further knowledge of the complexity of the CMBs in SSA, brought to light through sequencing data obtained from the last decade of studies, indicates even greater genetic diversity among EACMV species and strains than was earlier envisaged (Fauquet *et al*., 2008).

Development of multiplex PCR, a technique for detecting multiple viruses by combining multiple primer sets into a single amplification reaction (Deb and Anderson, 2007) enabled the simultaneous detection of ACMV and EACMCV for the first time in cassava (Alabi *et al*., 2008), and multiple viruses in other plant species (Nie *et al*., 2000; Bertolini *et al*., 2001; Deb and Anderson 2007; Roy *et al*., 2010; Hu *et al*., 2010). More recently, Abarshi *et al*. (2012) developed reverse transcriptase (RT) multiplex PCR tool for the simultaneous detection of RNA and DNA viruses co-infecting cassava. The RT multiplex PCR tool reliably detected the two cassava brown streak associated viruses, *Cassava brown streak virus* (CBSV) and *cassava brown streak Uganda virus* (CBSUV), although it weakly distinguished RNA and DNA cassava viruses.

Currently, there are more CMBs affecting cassava in SSA than were detected by the Alabi *et al*. (2008) multiplex PCR. Therefore there remains a great need for a more specific, sensitive and reliable diagnostic tool to distinguish the major CMBs in SSA. This study aimed to develop a more specific, sensitive and reliable single duplex and multiplex PCR tool for the simultaneous detection of four major CMB species in east and southern Africa.

2.3. Materials and Methods

2.3.1. Collection of CMD virus isolates

A total of 172 cassava leaf samples showing virus and virus-like symptoms of CMD were collected from cassava fields in Kenya (20), Malawi (32), Mozambique (20), Rwanda (20), Tanzania (60) and Zambia (20) between 2010 and 2011. The samples were pressed between papers packed in envelopes and shipped to Mikocheni Agricultural Research Institute (MARI), Tanzania where they were stored in a dry place until DNA extraction and analysis.

2.3.2. DNA extraction

Extraction of DNA was conducted using a modified SDS-based extraction protocol of Dellaporta *et al*. (1983). In the modified protocol, liquid nitrogen was excluded in DNA extraction process; 50 mg of dry leaf were directly ground in 700 µls of extraction buffer contained 700mM $NaCl_2$ and 20mM of βeta-mercaptoethanol. Other steps remained the same except the final DNA pellets were washed into 700 µls of wash buffer (75% ethanol

and 10 mM sodium acetate) instead of 80% ethanol for Dellapotra *et al*. (1983). The modified extraction protocol was used to extract DNA using both freshly-collected and dry leaf samples. Procedures for DNA extraction were similar, except more fresh leaf (100 mg) was used. DNA qualities were checked on 1% agarose gel and the quantity estimated relative to known concentrations of lambda DNA (NEB N3011S, New England Biolabs, Ipswich, MA).

2.3.3. Designing and screening novel primers for CMBs

Published full sequences of *ACMV EACMV, EACMCV, EACMKV, EACMMV* and *EACMZV* species available in the GenBank were used to design novel primers for use in this study **(Table 3).** The reference sequences were aligned using computer software packages MegAlign of DNAstar and MEGA4 (Tamura et al., 2007) and conserved regions in the DNA-A component specific to virus species selected for designing the novel primers. Specificity of the new primers to target virus species was confirmed by BLAST analyses of the sequenced PCR products.

Different primer pairs for multiplex PCR were designed such that their amplicons differed by at least 60 bp. This allowed for separation of amplicons and discrimination of the CMBs in agarose gel electrophoresis. A total of twelve (12) sets of virus specific primers were designed from published full sequences of ACMV and EACMV species to target amplification of various regions of the DNA-A component of the CMBs **(Table 3).** The length of the primers ranged between 18-25 base pairs (bp) with a melting temperature (TM) of 57 ± 5^{0}C. Primers were screened empirically for amplification of their targets. Negative controls were obtained from virus-free tested in vitro cassava

plantlets of clone TME7 obtained from ETH (Zurich, Switzerland) and maintained in the tissue culture laboratory at Mikocheni Agricultural Research Institute (MARI), Dar es Salaam, Tanzania. Positive controls for the CMBs were obtained from clones of respective viruses whose identity was previously confirmed by sequencing.

Table 3: List of primers used for amplification cassava mosaic begomoviruses in uniplex, duplex and multiplex PCR

Primer name	Sequence (5'→ 3')	Virus species	Target region	Expected size (nt)	Reference
JSP001	ATGTCGAAGCGACCAGGAGAT				Fondong *et. al.*, 2000
JSP002	TGTTTATTAATTGCCAATACT	ACMV	AV1/CP	554	
EAB555/F	TACATCGGCCTTTGAGTCGCATGG				Fondong *et. al.*, 2000
EAB555/R	CTTATTAACGCCTATATAAACACC	CMBs	DNA B	744	
ACMV1[a]	GTGGGCCTGGGCTGACACAC				
ACMV2 [a]	GCGTAGGAGAGTGGATCTTGTC	ACMV	DNA A	948	This study
EACMKV1 [b]	AAGGAGTCAGAGGCTCTTG				
EACMKV2[b]	CCACGTTTGAATTTCAAATTC	EACMKV	DNA A	669	This study
EACMMV1 [c]	GTGCCCTGTTCTTCACGGT				
EACMMV2 [c]	ACACACGTCCCAGACGAAA	EACMMV	DNA A	503	This study
EACMCV1 [d]	AAGTCTGAGGATGTAAACGAG				
EACMCV2 [d]	ACCTAGACGAGGACAAGAATTCC	EACMCV	DNA A	435	This study
EACMV1 [e]	GTTCGGCTATCACCTTCTAGAACA				
EACMV2 [e]	CAAGGCTTACATTGAAAAGGGA	EACMV	DNA A	375	This study
EACMZV1 [f]	CCAGGTCGAAGAATCGCTTA				
EACMZV2 [f]	AGGTGTCTCCAATTGCTCTC	EACMZV	DNA A	260	This study
EACMMV-F [c]	AACAAGCGACGATCATGGACGTTC				
EACMMV-R [c]	ACACACGTCCCAGACGAAA	EACMMV	DNA A	1630	This study
ACMV-F [a]	GAAGCACCTTGGTATCTGTAAGGTG				
ACMV-R [a]	CAAGAAGCGCTAAAGGCC	ACMV	DNA A	1106	This study
EACMZV-F [f]	GAAACATAAGGAGCTGGT				
EACMZV-R [f]	AGGTGTCTCCAATTGCTCTC	EACMZV	DNA A	575	This study
EACMV-F [e]	CCCCACAACATGCCCGCACT				
EACMV-R [e]	GGCCTTCACAGCCCTTCGGG	EACMV	DNA A	512	This study
EACMCV-F [d]	GGTAATGGGTTTAAGGACTGGT				
EACMCV-R [d]	CCTGGTTAGACAACTGCATATT C	EACMCV	DNA A	305	This study
EACMKV-F [b]	TTGTCCTCCTCGAGCAGATCGTC				
EACMKV-R [b]	AAGTCCTATATGGACAAGGAC	EACMKV	DNA A	238	This study
RBCL-F535	CTTTCCAAGGCCCGCCTCA	Rubisco L		171	Nassuth *et al.*, 2000
RBCL-R705	CATCATCTTTGGTAAAATCAAGTCC A				

[a]Primer designed using GenBank accession no. AF112352, AF259894, AF366902, AY795982, F126800, FN435276.
[b]Primer designed using GenBank accession no. AJ717582, AJ717577, AJ717571, AJ717569, AJ717578, AJ717581
[c]Primer designed using GenBank accession no. AJ006459, AJ006460,
[d]Primer designed using GenBank accession no. EU685323, EU685321, EU685319, EU685326, AF259896, AJ867444
[e]Primer designed using GenBank accession no. AJ717546, AJ717553, AJ006458, AY795986, Z83256, AJ717537
[f]Primer designed using GenBank accession no. AJ717567, AJ717564, AJ516003, AJ717563, AJ717560, AJ717583

2.3.4. Optimization of PCR conditions

Optimization of PCR conditions was conducted for the novel primers described in section 2.3. Key conditions optimized were annealing temperatures: 48^0C, 52^0C & 56^0C; template concentrations: 1 ng, 10 ng, 20 ng, 50 ng, 100 ng & 150 ng and primer concentrations: 0.1µM, 0.2µM & 4µM. The final PCR conditions for uniplex were performed at 50 µl PCR reaction containing a mixture of 0.6X PCR buffer, 1.25 mM of $MgCl_2$, 0.05 mM dNTPs, 0.2 µM each for forward and reverse primers, 1.5 U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany), 1 µl (20ng/µls) of DNA and 36.2 µl of sterilized distilled water.

Primers demonstrating efficient amplification in niplex and duplex PCR, were optimized in a multiplex PCR reaction containing a mixture of 1X PCR buffer, 2 mM of $MgCl_2$, 0.1 mM dNTPs, 0.2 µM of each forward and reverse primers, 1.5U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany), 3 µl (20 ng/µl) of DNA and sterile distilled water to make 50 µl PCR reaction mix. When a primer produced a too bright or too faint band, the concentration of the primer was decreased or increased, respectively to obtain a near similar amplification intensity.

2.3.5. Evaluation of specificity and sensitivity of the new primers

Specificity of each primer for uniplex, duplex and multiplex PCR (**Table 3**) to amplify target virus species was evaluated using artificially created mixed infections of total genomic DNA from leaf samples infected with: ACMV, EACMV, EACMCV, EACMKV, EACMMV and EACMZV.

Sensitivity of the new primers was evaluated by determining their detection limits in PCR in a 10-fold serially diluted DNA. In each detection tool, the primer pair: RBCL-F535 and RBCL-R705 **(Table 3)** targeting the cassava housekeeping gene [Ribulose biphosphate carboxylase oxygenase gene (Rubisco L)] (Nassuth *et al*., 2000) was run separately as an internal control.

PCR was run in a thermocycler (Gene Amp PCR system 9700, Singapore) using the following program: an initial denaturation step at 94^0C C for 3 min followed by 30 cycles at 94^0C for 30 sec, 52^0C for 30 sec, 72^0C for 1 min, and a final extension step at 72^0C for 7 min. A similar PCR program was used for uniplex, duplex and multiplex with an optimized annealing temperature of 52^0C for 30 sec. Ten microliters of the amplified products were electrophoresed in a 2% agarose gel containing (10mg/ml) ethidium bromide for 2 hrs and visualized under an UV transiluminator.

2.3.6. Validation of multiplex PCR detection of CMBs

Field-collected cassava leaf samples were initially screened for the presence of CMBs using primer JSP001/JSP002 for ACMV and the universal primer EAB555-F/EAB555-R for EACMV (Fondong *et al*., 2000). Detection of single viruses was achieved using the designed primers: ACMVI/2, EACMV1/2, EACMCV1/2, EACMKV1/2, EACMMV1/2 and EACMZV1/2 for ACMV, EACMV, EACMCV, EACMKV, EACMMV and EACMZV respectively **(Table 3).**

For the detection of more than one viral target in a single tube, duplex PCR was performed as described in section 2.4 with addition of a second primer pair. The mixed infected DNA template was used with adopted annealing temperature of 52^0C for 30 sec

and primer concentration reduced to 0.1 µM /0.2 µM. CMBs were detected in multiplex PCR using similar conditions optimized for uniplex and duplex PCR for ACMV, EACMCV, EACMKV, EACMMV and EACMZV.

Selected PCR amplicons were cloned and sequenced at BecA/ILRI, Nairobi, Kenya and the sequences compared with those of the reference CMBs in the Genbank to confirm the validity of the results obtained with the new multiplex PCR.

2.4. Results

2.4.1. DNA extraction protocols

Clear bands were achieved with the modified DS-based DNA extraction protocol using dry leaves and were comparable to those obtained for fresh leaves (**Figure 4**). Similarly, the concentration of DNA was comparable between the dry leaves and fresh leaves using the modified protocol. The average concentration of DNA recovered from 50 mg of dry leaf tissues as estimated relative to known concentration of lambda DNA was 116 µg/µl (**Table 4**). The resulting DNA was used successfully in uniplex, duplex and multiplex PCR amplification (**Figure 5 - 10**).

Furthermore, PCR assay using the housekeeping gene (Rubisco L) confirmed the presence of cassava DNA in all dry leaf samples extracted using the modified SDS extraction protocol (**Figure 9F**).

Table 4: Comparison of DNA yield obtained from fresh cassava leaves using modified and dry cassava leaves using unmodified SDS-based Dellaporta *et al*. (1983) extraction protocols

Sample no.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean**
Modified (ng/µl)	100[a]	180	80	170	100	180	80	150	60	60	**116**
Original (ng/µl)	220	280	260	200	180	80	60	200	100	300	**188**

[a]The quantities were estimated by comparing extracted DNA with serially diluted lambda DNA as standards in 1% agarose gel.

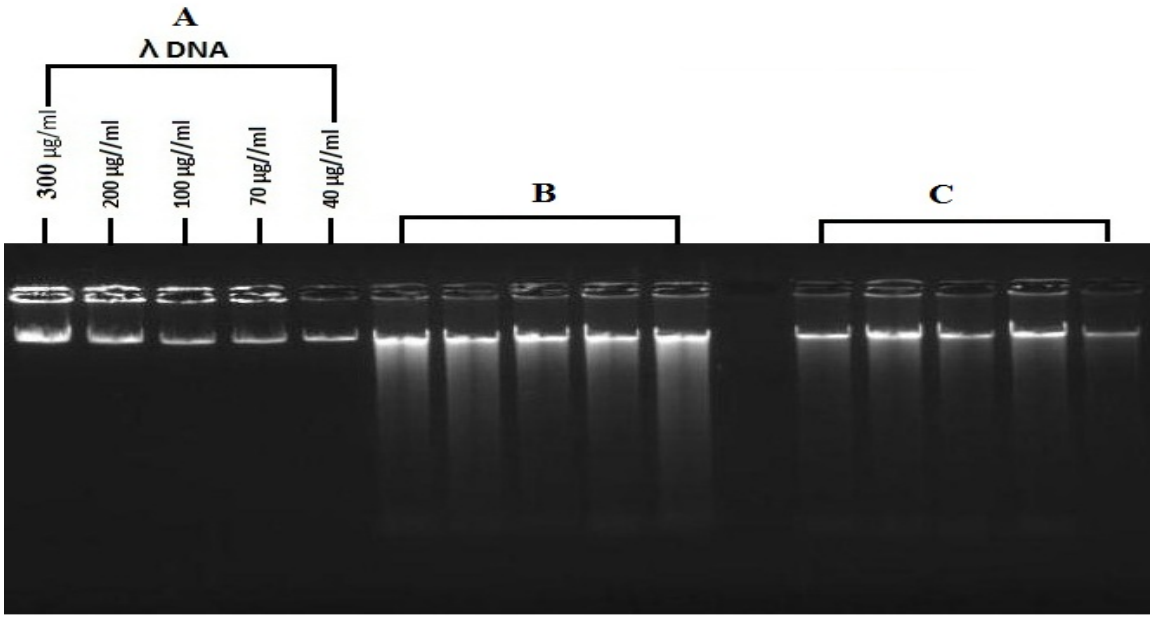


Figure 4: Agarose gel electrophoresis of DNA extracted from cassava leaves. **A:** Known concentrations of Lambda DNA used to estimate DNA yields; **B:** DNA isolated by SDS-based DNA extraction protocol (Dellaporta *et al*., 1983) using fresh cassava leaves; **C:** DNA isolated by modified SDS-based DNA extraction protocol from dry cassava leaves.

2.4.1 Optimization of PCR conditions

In uniplex PCR assays, results of the three annealing temperature regimes showed that at 48^0C, the majority of the primers produced a faint band for the detection of CMBs (Data not shown). When the temperature was raised to 52^0C all the primers produced good amplification of a single expected sharp amplicon band (**Figure 5**). A further raise of annealing temperature to 56^0C, majority of the primers **(Table 3)** produced unspecific multiple bands (data not shown). Consequently, annealing temperature of 52^0C was finally adapted for duplex and multiplex PCR.

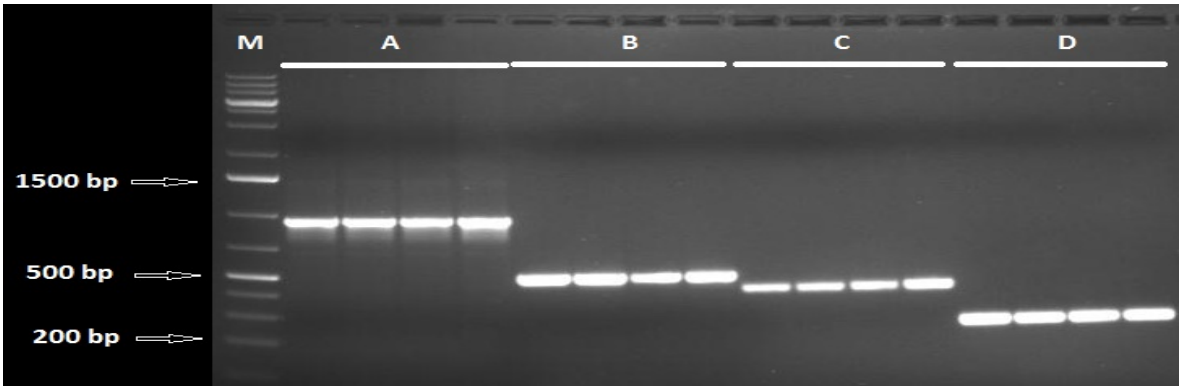


Figure 5: Amplification of the four cassava mosaic begomovirus species **A**: ACMV, **B:** EACMMV, **C:** EACMCV and **E:** EACMZV at 52^0C optimized annealing temperature.

Of the six DNA template concentrations tested 20, 50, 100 and 150ng produced similar band brightness across the four primer sets (**Figure 6A- D**). In contrast, the amplification efficiency decreased with less concentration from 10 to 1ng. The 10ng template was amplified for all the four viruses with lower intensity, while the 1ng template produced very faint bands with ACMV and EACMZV, and no amplification with EACMMV and

EACMCV (**Figure 6A-D**). Thus, optimum DNA template concentration was established at 20 ng. At this concentration all primers produced sharp bright bands (**Figure 6A-D**).

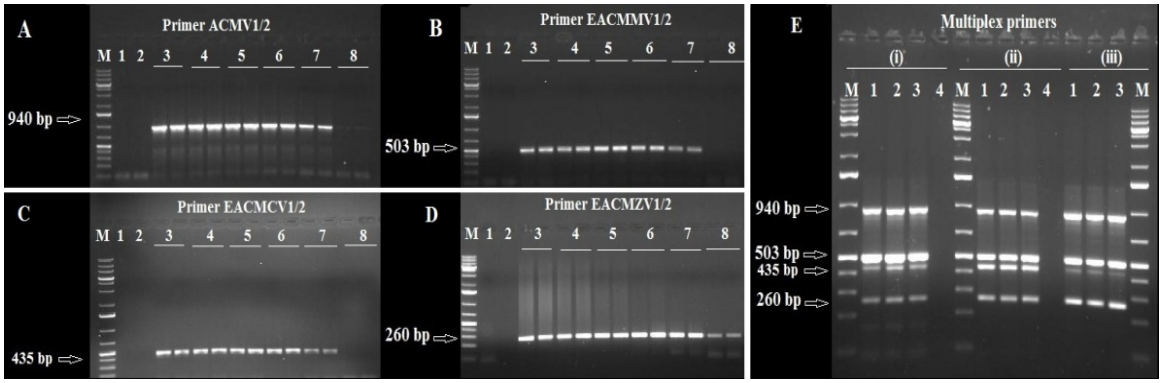


Figure 6: Determination of optimum template concentration for uniplex PCR amplification of **A:** ACMV; **B:** EACMMV; **C:** EACMCV and **D:** EACMZV using primers ACMV1/2, EACMMV1/2, EACMCV1/2, and EACMZV1/2, respectively. **Lane M:** 1 kb plus DNA ladder (*MBI, Fermentas, St. Leon-Rot, Germany*); **Lane 1:** negative control from a health plant DNA; **Lane 2:** negative water control; **Lane 3-8:** template concentration of 150ng, 100ng, 50ng, 20ng, 10ng and 1ng/ul, respectively. **E:** Optimization of primer concentration for cassava mosaic begomoviruses multiplex PCR analysis **(i):** Concentration at 0.2 µM; **(ii):** concentration of ACMV1/2 and EACMMV1/2 is 0.1 µM each, concentration of primers EACMCV1/2 and EACMZV1/2 is 0.2 µM each; **(iii):** concentration of ACMV1/2 and EACMMV1/2 is 0.1µM each, concentration of primer EACMCV1/2 is 0.2µM and concentration of EACMZV1/2 is 0.4µM.

2.4.2 Specificity of novel PCR primers

Uniplex PCR

To ensure robustness of a multiplex PCR, new primers were optimized to obtain the best combination. Primers were tested in uniplex and multiplex PCR to eliminate nonspecific reactions and effect of primer interactions before optimizing the final multiplex PCR. Of the twelve primers designed in this study (**Table 3**), four primer pairs: ACMVI/2, EACMCV1/2, EACMMV1/2 and EACMZV1/2 amplified the expected targets for ACMV, EACMCV, EACMMV and EACMZV, respectively (**Figure 7A-D**).

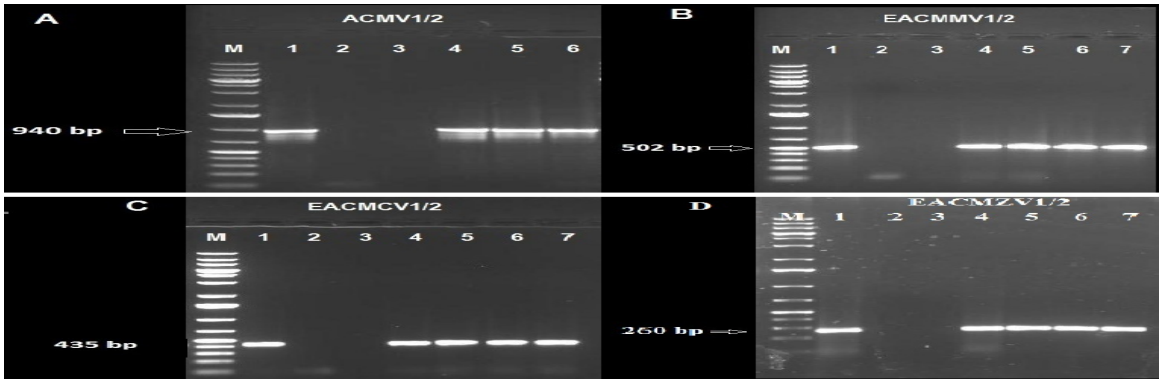


Figure 7 A- D: Screening of novel cassava mosaic begomovirus primers. **Lane M:** 1 kb plus DNA ladder (*MBI, Fermentas, St. Leon-Rot, Germany*); **Lane 1:** positive control; **Lane 2:** negative control from a health plant DNA; **Lane 3:** negative water control; **Lanes 4 to 7:** virus infected field sample

Duplex PCR

Based on the results obtained in section 3.3 the four successful primers ACMVI/2, EACMMV1/2, EACMCV1/2 and EACMZV1/2 were selected for subsequent studies. The ability of the new primers to simultaneously detect more than one virus was tested, using known CMB DNA samples of virus combinations for ACMV+EACMMV, ACMV+EACMCV, ACMV+EACMZV, EACMMV+EACMCV, EACMMV+EACMZV and EACMCV+EACMZV in duplex PCR. In each duplex PCR involving ACMV and any one of the three EACMVs, two bands were obtained (**Figure 8A-C**). Primer combination ACMV1/2+EACMMV1/2 amplified two bands of 948 bp specific to ACMV and 503 bp specific to EACMMV. Similarly, primer combination ACMV1/2+EACMCV1/2 gave two bands of 948 bp specific to ACMV and 435 bp for EACMCV, while primer combination ACMV1/2+EACMZV1/2 amplified two bands 948 bp and 260 bp specific for ACMV and EACMZV, respectively (**Figure 8A- C**). On the

other hand, duplex PCR involving CMBs EACMMV+EACMCV, EACMMV+EACMZV and EACMCV + EACMZV (**Figure 8D-F**), primer combinations EACMMV1/2 +EACMCV1/2, EACMMV1/2 + EACMZV1/2 and EACMCV1/2 + EACMZV1/2 amplified two bands each of 504 & 435 bp, 504 & 260 bp and 435 & 260 bp, respectively.

Multiplex PCR

In multiplex PCR, the four CMB species were also successfully detected from artificially created multiple infected samples using equal concentrations of DNA of individual virus species for ACMV, EACMCV, EACMMV and EACMZV. The viral species-specific primers: ACMV1/2, EACMCV1/2, EACMMV1/2 and EACMZV1/2 simultaneously amplified four virus species with similar intensity as in uniplex and duplex PCR **(Figure 8G).**

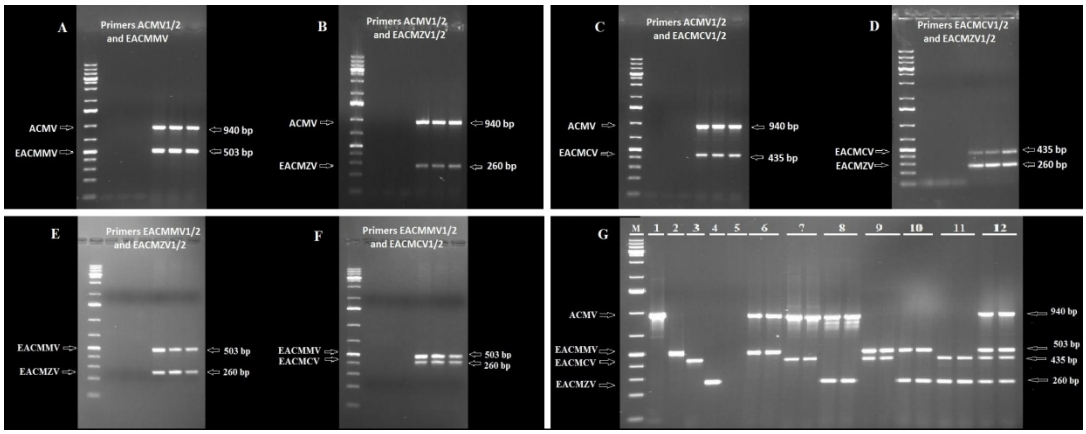


Figure 8: Duplex PCR analysis for the simultaneous detection of cassava mosaic begomoviruses (CMBs) from known virus-infected samples using primer combinations: **A:** ACMV1/2 & EACMMV1/2; **B:** ACMV1/2 & EACMZV1/2 &; C: ACMV1/2 & EACMCV1/2; **D:** EACMCV & EACMZV1/2; **E:** EACMMV & EACMZV and **F:** EACMCV & EACMZV designed in this study. **Lane M:** 1 kb plus DNA ladder (*MBI, Fermentas, St. Leon-Rot, Germany*); **Lanes 1:** no template negative control; **Lane 2&3:** negative control from health plants; **Lanes 4-6:** known virus samples. **G:** Uniplex, duplex

and multiplex PCR assays for detection of CMBs using the new primers. **Lane 1-4:** DNA extracts with single virus targets; lane 5: negative control; **Lanes 6-11:** DNA extracts with double virus targets; **Lane 12:** DNA extract with combination of virus targets for ACMV, EACMMV, EACMCV, EACMKV and EACMZ.

Of the four CMBs amplified, ACMV and EACMCV were readily detected by their specific primers ACMV1/2 and EACMCV1/2, respectively. Also primer EACMMV1/2 and EACMZV1/2 amplified the expected specific PCR products for EACMMV and EACMZ, respectively. Amplification intensity differed between primer combinations for multiplex PCR at 0.2µM primer concentration (**Figure 6Ei**). Optimum amplification was obtained when concentrations of primers ACMV1/2 and EACMMV1/2 were reduced to 0.1 µM, while the concentration of EACMCV1/2 and EACMZV1/2 were maintained at 0.2 µM (**Figure 6Eii**). Maintaining concentrations of primers ACMV1/2 and EACMMV1/2 at 0.1 µM each, and EACMCV1/2 at 0.2 µM while increasing that of EACMZV to 0.4 µM, reduced amplification of EACMCV (**Figure 6Eiii**).

Representative PCR products amplified by primers for ACMV, EACMCV, EACMMV and EACMZV were gel-eluted and cloned into pGEM-T-Easy vector and their nucleotide sequences determined. Alignment of consensus sequences with reference sequences of the CMBs in the GenBank (**Table 5**) revealed a homology of 94–99%.

Table 5: Comparison of sequence identities of gel-eluted PCR products amplified by the new uniplex, duplex and multiplex primers designed in this study with the sequences of the corresponding regions from the Genbank.

Isolate name	Target sequence	Primer used	% Identity	Virus match in the genbank	Genbank Accession No.	Reference
A1	ACMV	ACMV1/2	97	ACMV	AM502340	Sserubombwe *et al*., 2008
A2	ACMV	ACMV1/2	94	ACMV	AM502338	Sserubombwe *et al*., 2008
A3	ACMV	ACMV1/2	98	ACMV	JN053430	Ramkat *et al.*, 2011
C1	EACMCV	EACMCV1/2	95	EACMCV	AY795984	Ndunguru *et al.*, 2005
C2	EACMCV	EACMCV1/2	98	EACMCV	AY795984	Ndunguru *et al.*, 2005
C3	EACMCV	EACMCV1/2	97	EACMCV	AY795984	Ndunguru *et al.*, 2005
Z1	EACMZV	EACMZV1/2	97	EACMZV	AJ717567	Bull *et. al.*,2006
Z2	EACMZV	EACMZV1/2	98	EACMZV	AJ717568	Bull *et. al.*,2006
Z3	EACMZV	EACMZV1/2	99	EACMZV	AJ516003	Were *et. al.*, 2004
M1	EACMMV	EACMMV1/2	99	EACMMV	AJ006459	Zhou *et. al.*, 1997
M2	EACMMV,	EACMMV1/2	99	EACMMV	AJ006459	Zhou *et. al.*, 1997
M5	EACMMV	EACMMV1/2	99	EACMMV	AJ006459	Zhou *et. al.*, 1997

2.4.3 Sensitivity of uniplex and multiplex PCR

Detection limits of the uniplex and multiplex PCR were tested in 10 folds (10^{-1} to 10^{-6}) serial dilutions. Positive results were obtained in uniplex PCR at the dilution of up to 10^{-4} with all four primers for ACMV, EACMCV, EACMMV and EACMZV (**Figure 9A-D**). In contrast, only faint PCR products were obtained with the 10^{-5} dilution for EACMMV and EACMZV primers, while no amplification was obtained at 10^{-6} dilution (**Figure 9A-D**). In multiplex PCR, positive results were observed only for a dilution of up to 10^{-3} for all the four CMBs (**Figure 9E**). In the Rubisco PCR assay, amplification of a 171 bp amplicon specific to Rubisco L gene from cassava genome was successful up to a dilution of 10^{-5} (Figure 9F). No amplification was obtained in uniplex, multiplex and even for Rubisco PCR beyond the 10^{-5} d**il**ution (**Figure 9A-F**).

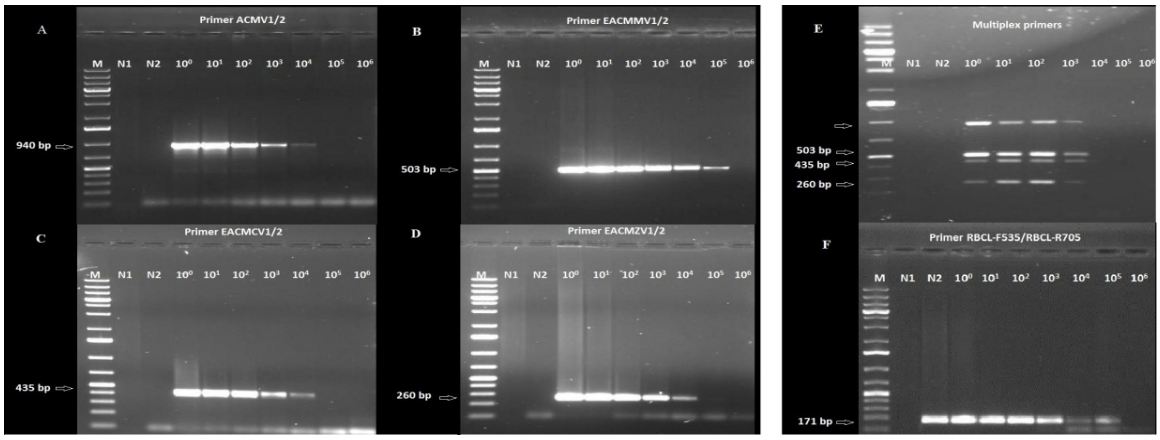


Figure 9: Determination of primer sensitivity limits in amplification of serially diluted DNA for the detection of **A:** ACMV; **B:** EACMMV; **C:** EACMCV; **D:** EACMZV; **E:** multiple infected virus samples (ACMV, EACMMV, EACMCV, EACMKV and EACMZV) and **F:** internal control RubiscoL gene. In A-F **Lane M:** 1 kb plus DNA ladder (*MBI, Fermentas, St. Leon-Rot, Germany*); **Lane 1:** no virus water control; lane 2: negative control from a health plant DNA; lane 3-9: 10-fold serially diluted (10^{-1}-10^{-6}) DNA samples, with lane 3 representing original extracts. PCR amplicon sizes are indicated in the left hand side.

2.4.4 Validation of multiplex PCR in detection of CMBs from field-collected samples

Validation of multiplex PCR for the detection of ACMV, EACMCV, EACMMV and EACMZV in single and mixed infection was conducted using Fondong et al. (2000) and the new multiplex primers using 172 diseased samples collected from farmers' fields from 6 countries (**Table 6A & B**, **Figure 10A-C**). Using Fondong *et al*. (2000) universal primers that only distinguish ACMV and EACMV and not the EACMV species, ACMV and EACMV were detected in 37 (24.8%) and 126 (84.6%) of the samples, respectively.

Table 6A: Detection of ACMV and EACMV-like viruses using the universal primers JSP001/002 and EABB555-F/R in uniplex PCR

Country	Total no. of samples	ACMV alone	EACM-like viruses	ACMV & EACM-like viruses (Co-infection)	No amplification samples
Kenya	20	2 (10%)	18(90%)	0(0%)	0 (0%)
Tanzania	60	11(19.0%)	39(67.2%)	8(13.7%)	2(3.4%)
Malawi	32	0(0.0%)	25(100%)	0(0.0%)	7(28%)
Zambia	20	6(40%)	9(60%)	0(0%)	5(25%)
Mozambique	20	1(7.7%)	12(92.3%)	0(0%)	7(35%)
Rwanda	20	3(16.7%)	9(50%)	6(33.%)	2(10%)
TOTAL	**172**	**23 (13.4%)**	**112 (65.1%)**	**14 (8.1%)**	**23 (13.4%)**

Table 6B: Detection of ACMV, EACMMV, EACMCV and EACMZV using primers ACMV1/2, EACMMV1/2, EACMCV1/2 and EACMZV1/2 in multiplex PCR

Cassava mosaic Begomoviruses	Kenya	Tanzania	Malawi	Zambia	Mozambique	Rwanda	Total
ACMV	0(0%)	8(7.7%)	0(0%)	6(5.8%)	0(0%)	6(5.8%)	20(19.2%)
EACMMV	0(0%)	4(3.8%)	7(6.7%)	0(0%)	0(0%)	0(0%)	11(10.6%)
EACMCV	0(0%)	3(2.9%)	3(2.9%)	0(0%)	0(0%)	1(0.9%)	7(6.7%)
EACMZV	10(9.6%)	8(7.7%)	5(4.8%)	0(0%)	9(8.7%)	0(0%)	32(30.8%)
ACMV+EACMCV	0(0%)	3(2.9%)	0(0%)	0(0%)	0(0%)	3(2.9%)	6(5.8%)
ACMV+EACMZV	3(2.9%)	7(6.7%)	0(0%)	0(0%)	0(0%)	0(0%)	10(9.6%)
EACMMV+EACMCV	0(0%)	1(0.9%)	2(1.9%)	0(0%)	0(0%)	0(0%)	3(2.9%)
EACMMV+EACMZV	0(0%)	2(1.9%)	5(4.8%)	0(0%)	0(0%)	2(1.9%)	9(8.7%)
EACMCV+EACMZV	0(0%)	1(0.9%)	2(1.9%)	0(0%)	0(0%)	0(0%)	3(2.9%)
ACMV+EACMMV+EACMZV	0(0%)	0(0%)	1(0.9%)	0(0%)	0(0%)	0(0%)	1(0.9%)
ACMV+EACMCV+EACMZV	0(0%)	2(1.9%)	0(0%)	0(0%)	0(0%)	0(0%)	2(1.9%)
NO AMPLIFICATION	7(4.1%)	21(12.2%)	7(4.1%)	14(8.1%)	11(6.4%)	8(4.7%)	68(39.5%)
Total	**20**	**60**	**32**	**20**	**20**	**20**	**172**

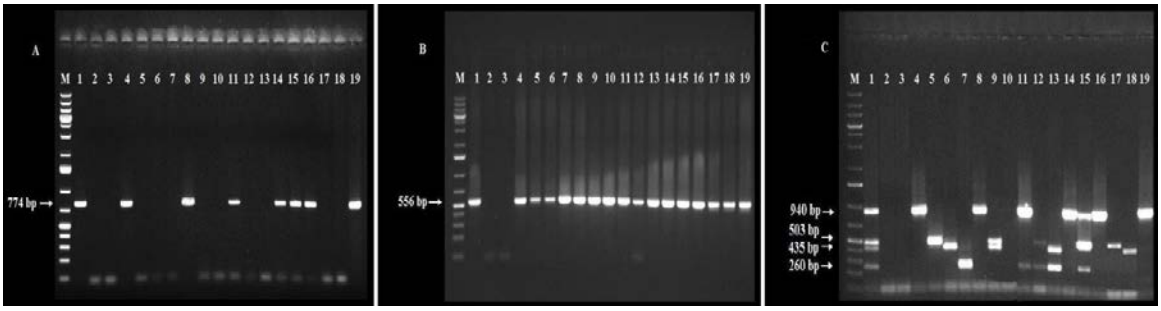


Figure 10: Comparison between A: uniplex PCR using primer JSP001/JSP002 (Fondong *et al*., 2000) for detection of ACMV; **B:** uniplex PCR using universal primers EABB555-F/EAB555-R (Fondong *et al*., 2000) for detection of all species of EACMV and C: multiplex PCR for detection of ACMV, EACMMV, EACMCV and EACMZV. In A to C, **Lane M:** 1 kb plus DNA ladder (*MBI, Fermentas, St. Leon-Rot, Germany*); **Lane 1**: positive control; **Lane 2:** negative control from a health; **Lane 3:** no extract negative control; **Lanes 4-20:** DNA extracts from field samples collected in Malawi and Rwanda.

Co-infection of ACMV and EACMV was detected in 14 (9.4%) of the samples. However, 23 (13.4%) of the samples tested negative for both ACMV and EACMV. Using the new multiplex PCR primers, all four CMB species were amplified **(Table 6B).** The PCR results were confirmed by sequencing. Of the 172 samples, 104 (60.5%) were infected with CMBs (**Table 6B**). Generally, EACMZV was the most frequently detected virus of the four CMBs and it occurred in 57 (54.8%) samples. This was followed by ACMV, EACMMV and EACMCV, which occurred in 39 (37.5%), 24 (23.1%), 21 (20.2%) of the samples, respectively.

All together, co-infections of ACMV and EACMVs were detected in 34 (32.7%) of the samples. ACMV+EACMCV, ACMV+EACMZV, EACMMV+EACMCV, EACMMV+EACMZV, EACMCV+EACMZV, ACMV+EACMMV+EACMZV and ACMV+EACMCV+EACMZV occurred in 6 (5.8%), 10 (9.6%), 3 (2.9%), 9 (8.7%), 3 (2.9%), 1 (0.9%) and 2 (1.9%) for CMBs, respectively (**Table 6B**). Detection of CMBs by multiplex PCR revealed Tanzania and Rwanda to have the highest virus diversity with

all four CMB species. Malawi and Kenya had three and two CMBs each, respectively. In contrast, Mozambique and Zambia each had only one CMB species (**Table 6B**).

Comparison of uniplex and multiplex PCR analysis of the CMBs occurring in the six countries using the universal primers of Fondong *et al*. (2000) for ACMV (JSP001/002) and EACMVs (EAB555F/R) demonstrated more positive reactions (149/172) than the new multiplex PCR (104/172). The EAB555F/R primers detected 112 EACMV positive samples, while multiplex PCR detected only 84 samples with EACMVs. However, multiplex PCR detected more ACMV samples (39) than the JSP001/002 primers (37).

2.5 Discussion

In the present study I reported for the first time a single-tube duplex and multiplex PCR for the simultaneous detection of four cassava mosaic begomoviruses: ACMV, EACMCV, EACMMV and EACMZV that are prevalent in cassava in Kenya, Malawi, Mozambique, Rwanda, Tanzania and Zambia. The new multiplex assay using primers designed in this study clearly distinguished the four CMBs in artificially created single and mixed infections and the results corroborated those obtained with the field-collected samples.

Primers in common use for CMB diagnostics such as JSP001/002 and EAB555F/R (Fondong *et al*., 2000) detect ACMV and EACMVs without discriminating the EACMV species. The first multiplex PCR enabled the simultaneous detection of only two CMBs, namely ACMV and EACMCV (Alabi *et al*., 2008). However, current knowledge of the viruses causing CMD in SSA shows a wide diversity of species to be involved (Fauquet *et al*., 2008). Further, diverse symptoms are expressed on CMD-affected plants in the

field, which range from mild to severe leaf distortions. The diverse field symptoms may be caused, among other factors, by plant varietal differences (host resistance), virus species/strains and nature of infection (single or mixed) (Gibson and Otim-Nape, 1997). For example, the dual infections of ACMV and EACMCV in Cameroon were associated with more severe symptoms than the single infections of the two viruses, which were attributed to a synergistic interaction (Fondong *et al*., 2000). Similarly, dual infections of ACMV and EACMV-Ug were reported to be responsible for the severe CMD pandemic in eastern and central Africa (Legg *et al*., 2011). Therefore the current situation warrants the development of diagnostic assays with the ability to detect even more CMB species causing the disease in SSA.

Our new multiplex PCR reliably distinguished single infections of ACMV, EACMCV, EACMMV and EACMZV, dual infections of ACMV & EACMCV, ACMV & EACMZV, EACMMV & EACMCV, EACMMV & EACMZV and EACMCV & EACMZV, and the triple infections of ACMV + EACMCV + EACMZV and ACMV + EACMMV + EACMZV. This shows that the new multiplex PCR is more versatile and robust than previously reported assays. It is suitable for use in diagnostic studies that require the specific detection of the CMBs causing disease on cassava. Moreover, knowledge is still lacking on the effect of multiple infections of EACMVs on the disease symptoms, growth and yield of cassava plants. A management practice that could exploit this knowledge may include the deployment of CMD-tolerant cassava cultivars with mild symptoms in the low disease pressure areas. Previous studies in Uganda indicated that mildly symptomatic plants of CMD-tolerant local cultivars were selected by small-holder

farmers for use in subsequent plantings because they produced comparable yield to healthy plants (Thresh *et al*., 1998).

Detection of templates with low virus titre as obtained in the dilution tests shows that the new assay is very sensitive. Recently, an RT-PCR protocol optimized for detection of CBSV and CBSUV amplified the target viruses up to a dilution of $1.5x10^{-3}$ (Alabi *et al*., 2008). Results obtained by our modified SDS-based DNA extraction protocol for uniplex PCR, showed more sensitivity in detecting all four CMBs in DNA samples diluted up to 10^{-4}, but near similar sensitivity for detecting the CMBs simultaneously in multiplex PCR for samples diluted up to 10^{-3}. Therefore the new assay has the advantage to detect CMBs in very low concentrations, as is often the case in some field samples. Of the four primers developed in this study, primer pair ACMV1/2 preferentially detected the target virus in uniplex, duplex and multiplex PCR than did the remaining three primers. This may probably be due to differences in viral sequences between ACMV and the EACMVs. A similarity of less than 70% between ACMV and the EACMVs resulted in less competition for primer annealing, enabling ACMV to be detected more readily in mixed infections with the EACMV species (Fauquet *et al*., 2008).

I also report here a modified Dellaporta *et al*. (1983) protocol, which was used to extract DNA from dry and fresh cassava leaves with comparable results. Modifications were made on the SDS-based DNA extraction protocol of Dellaporta *et al*. (1983) to yield high quality DNA from dry-stored cassava leaf samples. The modification excluded the use of liquid nitrogen during extraction. Although the original SDS-based DNA extraction protocol yields high quality DNA, its requirement for fresh leaf samples and use of liquid nitrogen make it expensive when used extensively to test multiple samples. Moreover,

liquid nitrogen is not easily available in the developing world. Further, samples are often collected from remote areas and delivered to a central laboratory for analysis after several days, leading to loss of integrity of the fresh leaves and the quality of DNA.

The modified SDS-based DNA extraction protocol gave good quality DNA that was suitable for sensitive detection. Exclusion of liquid nitrogen and direct grinding of the leaf tissues into extraction buffer significantly reduced the time and cost of DNA extraction. Interestingly, there was no noticeable degradation of DNA due to oxidation or other causes during extraction. The method was shown to work well for the detection of CMBs from dry stored cassava leaf samples. It is now possible to extend areas to be sampled during surveys, without the necessity of moving around with cool boxes and returning to the laboratory to store the fresh samples in -80^0C freezers. Care should taken during sampling to place the leaf samples between papers/or book pages and to keep them in dry conditions to avoid disintegration.

In conclusion, the new multiplex PCR reported here is most suitable for rapid diagnostic studies requiring the specific detection and identification of CMBs in field-collected samples without the need for sequencing. Using the duplex and multiplex techniques, time was saved and amount of reagents used were reduced, which translated into reduced cost of the diagnostics. I recommend the use of the multiplex PCR assay for rapid and extensive leaf sampling for cassava breeders screening for disease resistance, scientists doing virus diagnostic studies, phytosanitary officers checking movement of diseased planting materials, and seed certification and multiplication officers for virus indexing.

Acknowledgements

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CHAPTER THREE

IDENTIFICATION OF *CASSAVA BROWN STREAK VIRUS* SPECIES BY RT-PCR/RFLP ANALYSIS OF THE COAT PROTEIN CODING REGION

Manuscript submitted to Plant Disease.

3.1 Abstract

A reverse-transcriptase polymerase chain reaction/restriction fragment length polymorphism (RT-PCR/RFLP) was developed and successfully employed for the detection and identification of two species of cassava brown streak viruses (CBSVs), namely *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV). A degenerate primer pair amplifying a 752-785 bp fragment of the coat protein (CP) region was designed and used for RT-PCR, and the RT-PCR products digested with *Eco*RI and *Hind*III. The results were compared with other assays developed for detection of CBSVs. Digestion of RT-PCR products with *Eco*R1 endonuclease produced one fragment (785 bp) for CBSV and two fragments (528 and 224 bp) for CBSUV in single infected leaf samples. *Hind*III digestion yielded three fragments (437, 267 and 81 bp) for CBSV and one fragment (752 bp) for CBSUV in single infected samples. *Eco*RI digestion of RT-PCR products resulted in three DNA fragments (785, 528 and 224 bp), and *Hind*III produced four fragments (785, 437, 267 and 81 bp), from mixed infections of CBSV and CBSUV.

Comparison of RT-PCR/RFLP results and other PCR based assays showed similar results. Thus, the RT-PCR/RFLP assay may be a useful cost effective approach for

screening cassava samples on a large scale for the presence of CBSV and CBSUV. The assay may be used as a confirmatory test in virus indexing, CBSVs diversity studies and screening for disease resistance which information will be used for monitoring cassava viruses and advising management decisions.

3.2 Introduction

Cassava (*Manihot esculenta* Crantz, family *Euphorbiaceae*), is the second most important food security crop after maize, providing more than half of dietary calories for a majority of both the rural and urban populations in sub-Saharan Africa (SSA) (Abarshi *et al.*, 2010). It appeals to poor household communities due to its ease cultivation and performs well on marginal lands where other crops fail.

However, cassava productivity in SSA is constrained by both abiotic and biotic stresses. Among the biotic constraints, Cassava brown streak disease (CBSD) is the second major viral disease of cassava, particularly in east and central Africa (Monger *et al.*, 2001a, Alicai *et al.*, 2009, Mbanzibwa *et al.*, 2009a and Winter *et al.*, 2010).

CBSD is caused by two phylogenetically distinct viral species with positive single-stranded RNA genomes, *Cassava brown streak virus* (CBSV) and/or *Cassava brown streak Uganda virus* (CBSUV), both members of the family *Potyviridae*, genus *Ipomovirus (*Monger *et al.*, 2001a*;* Winter *et al.*, 2010; Mbanzibwa *et al.*, 2009a). In this study, the two virus species collectively are referred to as CBSVs.

CBSD induces yellow chlorosis on the veins and general blotchy chlorotic mottle on the aerial part of the cassava plant (Nichols, 1950), and brown necrotic streaks/lesions easily visible on the green portions of stems. CBSD also causes necrosis of the edible storage roots, and renders them unfit for human and livestock consumption. Yield losses of up to 100% on susceptible varieties have been reported from CBSD (Hillocks *et al.*, 2001; Hillocks and Jennings, 2003).

CBSD diagnosis by visual observation of foliar and root symptoms expression on infected cassava plants is unreliable due to variability in patterns of symptoms expression between varieties and seasons. Immature leaves of infected cassava often appear symptomless (Monger *et al.*, 2011) and there are no distinctive differences in symptoms induced by CBSV and/or CBSUV, thus identification and differentiation of the two species by visual assessment is difficult.

Serological detection of CBSVs is now possible following development of Enzyme-linked immunosorbant assay (ELISA) kits that detect several isolates of CBSV (Winter et al., Personal communication). However, serological methods alone are inadequate in identifying and differentiating the two CBSV species since sensitivity of ELISA depends on adequate virus titers. Additionally, immature leaves of CBSVs-infected cassava appear symptomless, thus, ELISA can easily fail to detect CBSVs-infected cassava particularly those with mild or latent symptoms.

Reverse-transcriptase polymerase chain reaction (RT-PCR) is widely used in the detection of plant RNA viruses (Bustin, 2000; Mumford *et al.*, 2006). RT-PCR, when combined with restriction fragment length polymorphism (RFLP), becomes a more powerful technique to differentiate viruses (Tairo *et al.*, 2006; Berniak *et al.*, 2009). PCR/RFLP has been used successfully as an alternative method to identify various species of cassava-infecting begomoviruses without the need for sequencing (Bull *et al.*, 2006; Borah and Dasgupta, 2012).

CBSVs were initially detected using the RT-PCR with previously described CBSV10 & CBSV11 primers (Monger *et al.*, 2001), but the sensitivity of these primers to CBSVs

was limited even to known infected samples. (Mbanzibwa *et al.* (2011) further simplified the detection and discrimination of CBSVs species by developing a single RT-PCR assay that simultaneously detects both viruses in single and mixed infection. However, some of the CBSVs isolates still escape the detection with this assay when used alone.

More recently, Abarshi *et al. (*2012) improved and developed a diagnostic assay for the simultaneous detection and differentiation of CBSVs and CMBs, in a single tube. Although the assay simplified detection of the two CBSV viruses (CBSV and CBSUV), sensitivity was reduced when additional primers were added to include detection of CMBs). With the increased number of sequences available in the database to date (Mbanzibwa *et al.,* 2009, 2011b; Monger *et al.,* 2010; Winter *et al.,* 2010), further analysis of all full sequences of coat protein region available in the Genbank of CBSV and CBSUV was done, and unique restriction sites found, which could be used for differentiation of the two viruses by RFLP. Therefore, the aim of this study was to improve the diagnostic tool for identification and discrimination of CBSV and CBSUV in single and mixed infected cassava samples by designing a simple RT-PCR combined with RFLP.

3.3 Material and methods

3.3.1 Collection of plant materials for molecular analysis

A new procedure for collecting and storing dry samples in the field was employed. It involved use of paper packaging materials, which contained alternating hard and soft sheets arranged into a book (**Figure 11A**). Single leaflets with clear virus and virus-like symptoms of CBSD were picked from cassava plants in the field and placed between the

soft sheets (**Figure 11B**) in an ordered manner as they were collected (**Figure 11C**). To remove the moisture and preserve integrity of the leaves, the books were closed, pressed and kept in a clean dry place until laboratory analysis. After more than a month of storage, dry leaves stored using described procedure maintained much of their fresh leaf properties, including the greenish colour and disease symptoms, which could clearly be observed (**Figure 11D**). This procedure was used in this study to collect cassava leaf samples from farmer' fields in Mozambique (24), Malawi (24) and Uganda (24).

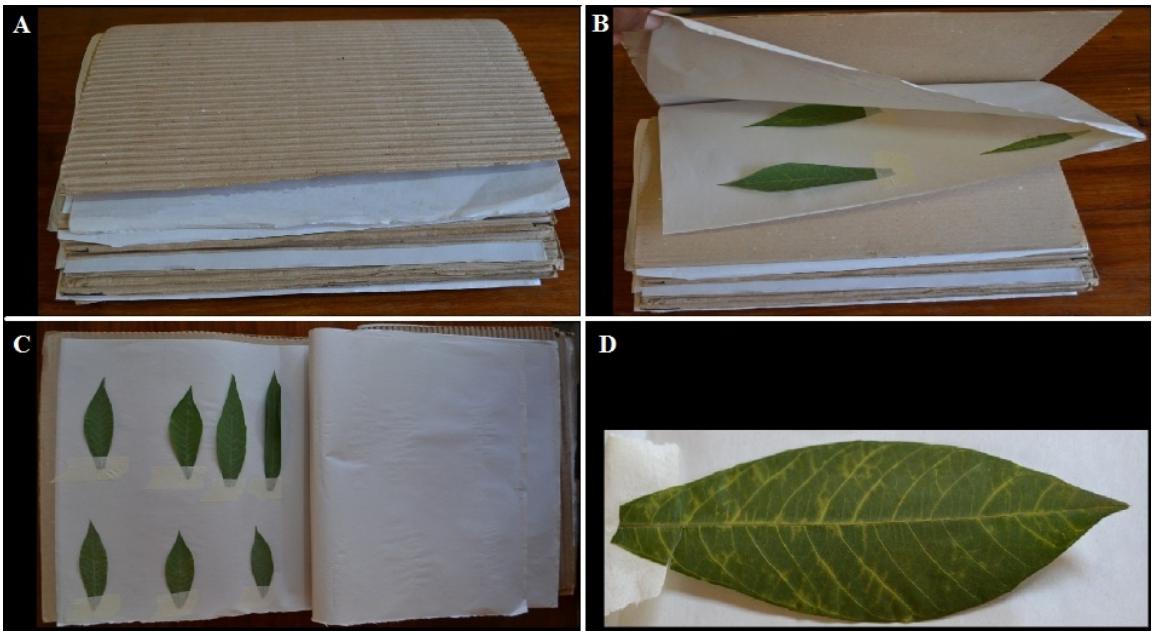


Figure 11: Diagrammatic representation of packaging and storage of sampled cassava leaves, **A-C:** Preparation of packaging material and arrangement of the stored leaves, **D:** Dry stored leaf showing preserved green colour and clear CBSD-like symptoms.

3.3.2 Isolation of RNA

RNA was extracted from cassava leaf samples using a modified Chang *et al.* (1993) cetyltrimethylammonium bromide (CTAB)-based protocol. Modification was made to the Chang *et al.*, (1993) method to reduce the time and cost of extraction without reducing

quality and yield of RNA extracted from dried leaves of cassava plants. In the modified protocol, liquid nitrogen was excluded in RNA extraction process; all centrifugation steps were carried out at 4°C. Fifty mg of dry leaf material was directly grounded in 700µl of extraction buffer contained 2% CTAB, and 2% βeta-mercaptoethanol and 2% PVP (the last two added just before use). Other steps were not changed except that RNA precipitation was carried out using 2 volumes of absolute ethanol instead of LiCl precipitation and incubated at -20°C for 5 minutes prior to RNA precipitation. RNA was extracted from both freshly collected and dry leaf samples using the modified protocol. In fresh leaf samples, RNA extraction procedures were similar except more fresh leaf (100mg) was used.

RNA quality was checked on a 2% agarose gel and the quantity estimated using a Nano drop instrument (Thermo Fisher Scientific Inc, USA). Comparison of RNA yield between dry and fresh leaves was determined by ANOVA of GenStat 14th edition from 35 DNA samples of each dry and fresh leaf extractions.

3.3.3 Primer designing and screening

Representative complete sequences of coat protein of CBSV and CBSUV were retrieved from the GenBank database (http://www.ncbi.nih.gov/) and used to design degenerate primers for universal amplification of the two CBSVs species. One degenerate primer pair was designed to amplify a 752-785 bp fragment in the conserved region of the CP gene of CBSV and CBSUV (**Table 7**). Using computer software packages MegAlign of DNAstar and MEGA4 (Tamura *et al.,* 2007) forward primer CPCBSV1 was designed from nucleotide (nt) position 165 downstream of the N-terminal of CP and reverse primer

CPCBSV2 was designed from the 950 nt position upstream the 3'-proximal part of CBSV and CBSUV.

Specificity of the designed primer pair to amplify CBSVs was tested using RNA of known CBSV and CBSUV positive samples. Specificity of the new primer was further confirmed by sequencing representative PCR products amplified by the designed degenerate primer. The negative controls were healthy cassava variety TME7, which was obtained from ETH (Zurich, Switzerland) and maintained at MARI biotechnology laboratory. Positive controls were obtained from clones of respective viruses whose identity were previously confirmed by sequencing. Optimum annealing temperatures for the new primer was determined from the TM range of 50 to 60°C and Tm of 58 to 60°C was chosen for this PCR amplification.

Table 7: List of isolates of Cassava brown streak virus (CBSV) and Cassava brown streak Uganda (CBSUV) viruses retrieved from GenBank used for designing primers.

Isolate name	Species	Geographical region	Restriction endonuclease		Accession no.	Reference
			***Eco*R1**	***Hind*III**		
Tan70	CBSV	Tanzania	785	437/267/81	FN434437	Winter *et al*., 2010
Mo_83	CBSV	Mozambique	785	437/267/81	FN434436	Winter *et al*., 2010
Tan Z	CBSV	Tanzania	785	437/267/81	GQ329864	Monger *et al*., 2010
Kor6:08	CBSV	Korogwe, Tanzania	785	437/267/81	GU563327	Manzibwa *et al*., 2011
Namp1:07	CBSV	Mozambique	785	437/267/81	HM346953	Manzibwa *et al*., 2011
Kar9:09	CBSV	Karonga, Malawi	785	437/267/81	HM171296	Manzibwa *et al*., 2011
Wak39:09	CBSV	Wakiso, Uganda	785	437/267/81	HM171313	Manzibwa *et al*., 2011
Wak33:09	CBSV	Wakiso, Uganda	785	437/267/81	HM171312	Manzibwa *et al*., 2011
ZANZ7-1	CBSV	Zanzibar	785	437/267/81	HM346958	Manzibwa *et al*., 2011
Naliendele3-1	CBSV	Naliendele, Tanzania	785	437/267/81	HM346954	Manzibwa *et al*., 2011
Kar17:09	CBSV	Karonga, Malawi	785	437/267/81	HM171319	Manzibwa *et al*., 2011
ZANZ11-1	CBSV	Zanzibar	785	437/267/81	HM346960	Manzibwa *et al*., 2011
Namp 1-1	CBSV	Nampula, Mozambique	785	437/267/81	HM346953	Manzibwa *et al*., 2011
ZANZ	CBSV	Zanzibar	785	437/267/81	GU563325	Manzibwa *et al*., 2011
PANG	CBSV	Pangani, Tanzania	785	437/267/81	GU563322	Manzibwa *et al*., 2011
ZANZ6-2	CBSV	Zanzibar	785	437/267/81	HM346956	Manzibwa *et al*., 2011
KOR9	CBSV	Korogwe, Tanzania	785	437/267/81	GU563324	Manzibwa *et al*., 2011
KOR1	CBSV	Korogwe, Tanzania	785	437/267/81	GU563320	Manzibwa et al., 2011
CHAKE	CBSV	Chakechake, Zanzibar	785	437/267/81	GU563326	Manzibwa *et al*., 2011
ZANZ8-2	CBSV		785	437/267/81	HM346957	Manzibwa *et al*., 2011
KOR1	CBSV	Korogwe, Tanzania	785	437/267/81	GU563320	Manzibwa *et al*., 2011
Chake chake	CBSV	Chake chake, Zanzibar	785	437/267/81	GU563326	Manzibwa *et al*., 2011
ZANZ8-2	CBSV	Zanzibar	785	437/267/81	HM346957	Manzibwa *et al*., 2011
HAND	CBSV		785	437/267/81	GU563321	Manzibwa *et al*., 2011
BSA4	CBSUV	Bushenyi, Uganda	528/224	752	EU916832	Mbanzibwa *et al*., 2009a
BSA2	CBSUV	Bushenyi, Uganda	528/224	752	EU916831	Mbanzibwa et al., 2009a
IGA8	CBSUV		528/224	752	EU916830	Mbanzibwa *et al*., 2009a
LWR2	CBSUV	Lwero, Uganda	528/224	752	EU916829	Mbanzibwa *et al*., 2009a
LWR2	CBSUV	Lwero, Uganda	528/224	752	EU916829	Mbanzibwa *et al*., 2009a
Ugandan	CBSUV	Uganda	528/224	752	FJ185044	Monger *et al*., 2010
Nam:04	CBSUV	Namulonge, Uganda	528/224	752	HM181930	Monger *et al*., 2010
Ke_125	CBSUV	Kenya	528/224	752	FN433930	Winter *et al*., 2010
Ke_54	CBSUV	Kenya	528/224	752	FN433931	Winter *et al*., 2010
Ma_42	CBSUV	Malawi	528/224	752	FN433932	Winter *et al*., 2010
Ma_43	CBSUV	Malawi	528/224	752	FN433933	Winter *et al*., 2010
Ug_23	CBSUV	Uganda	528/224	752	FN434109	Winter *et al*., 2010
Zom1:09	CBSUV	Zomba, Malawi	528/224	749	HM171300	Mbanzibwa *et al*., 2011
Rum27:09	CBSUV	Rumphi, Malawi	528/224	752	HM171299	Mbanzibwa *et al*., 2011
Nkhata: 29:09	CBSUV	Nkhata bay, Malawi	528/224	752	HM171303	Mbanzibwa *et al*., 2011
Kar10:09	CBSUV	Karonga, Malawi	528/224	752	HM171297	Mbanzibwa *et al*., 2011
Chu21:08	CBSUV	Chumani, Kenya	528/224	752	HM346950	Mbanzibwa *et al*., 2011
Kik10:08	CBSUV	Kikonde, Kenya	528/224	752	HM346947	Mbanzibwa *et al*., 2011
Den1:08	CBSUV	Denyenye, Kenya	528/224	752	HM346937	Mbanzibwa *et al*., 2011
Kil18:08	CBSUV	Kilifi, Kenya	528/224	752	HM346938	Mbanzibwa *et al*., 2011
Shi7:08	CBSUV	Shirazi,Kenya	528/224	752	HM346944	Mbanzibwa *et al*., 2011
Rak31:09	CBSUV	Rakai, Uganda	528/224	752	HM171311	Mbanzibwa *et al*., 2011
Wakiso 40:09	CBSUV	Wakiso, Uganda	528/224	752	HM171314	Mbanzibwa *et al*., 2011
Njule 16:04	CBSUV	Njule, Uganda	528/224	752	HM171315	Mbanzibwa *et al*., 2011
UG: TO4:04	CBSUV	Uganda	528/224	752	HM171316	Mbanzibwa *et al*., 2011

EBW60:04	CBSUV	Ebwana, Uganda	528/224	752	HM171317	Mbanzibwa *et al*., 2011
Kabanyoro4-3	CBSUV	Kanyoro, Uganda	528/224	752	HM346952	Mbanzibwa *et al*., 2011
Diani 3-1	CBSUV	Diani, Kenya	528/224	752	HM346941	Manzibwa *et al*., 2011
Nyumbsita5-4	CBSUV	Nyumba sita, Kenya	528/224	752	HM346942	Manzibwa *et al*., 2011
Kikonde11-5	CBSUV	Kikonde, Kenya	528/224	752	HM346946	Manzibwa *et al*., 2011
Mriana8-1	CBSUV	Mriana, Kenya	528/224	752	HM346945	Manzibwa *et al*., 2011

*Predicted size of RFLP patterns from the amplified 752-785bp fragment analyzed by computer program vector NTI using *Eco*RI and *Hind*III restriction endonucleases

3.3.4 cDNA synthesis and PCR amplification

First strand cDNA was synthesized using oligo$(dT)_{25}$ primer and 2 µg of total RNA in Moloney murine leukemia virus reverse transcriptase (MMLV-RT) according to manufacturer's instructions (MBI, Fermentas, St. Leon-Rot, Germany). PCR amplification was done on 50 µl, and the reaction contained a mixture of 0.6X PCR buffer, 1.25 mM of $MgCl_2$, 0.05 mM dNTPs, 0.2 µM of each forward and reverse primers (CPCBSV1 and CPCBSV2 respectively), 1.5U µl of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany), 3 µl of cDNA and 36.2 µl of sterilized distilled water. PCR was run in a thermocycler (Gene Amp PCR system 9700, Singapore) and program was carried out as previously described (Rajabu *et al.*, 2012). The PCR products were analyzed on 1% agarose gels stained with ethidium bromide (1 µl of 10 mg/ml in 100 ml gel) and photographed under UV light using UVP BioDoc-It imaging system.

Table 8: Primers used in this study

Primer name	Direction	Sequence (5'→ 3')	Virus species	Target region	Expected size	Reference
CPBSV1	Sense	CAAACAARDAARAGGCCRTG	CBSV & CBSUV	CP*		This study
CPBSV2	Antisense	TCGGCDAGRAARTCWATACC	CBSV & CBSUV	CP*	785	This study
CBSDDF2	Sense	GCTMGAAATGCYGGRTAYACAA	CBSV & CBSUV	CP*	344 bp for CBSV	Mbanzibwa *et al*., 2011
CBSDDR	Antisense	GGATATGGAGAAAGRKCTCC	CBSV & CBSUV	UTR**	438–440 bp for CBSUV	Mbanzibwa *et al*., 2011
CBSVF2	Sense	GGRCCATACATYAARTGGTT	CBSV & CBSUV	Ham1		Abarshi *et al*., 2012
CBSVR7	Antisense	CCCTTTGCAAARCTRAAATARC	CBSV & CBSUV	Ham1	345bp for CBSV	Abarshi *et al*., 2012
CBSVR8	Antisense	CCATTRTCTYTCCAMADCTTC	CBSV &	CP	440 bp for	Abarshi *et al*.,

			CBSUV		CBSUV	2012
PVD2-F	Sense	AYAGYGGGBAAYAGDCARCC		Cassava		
PVD2-R	Antisense	CTGAGCGTAAAGCAGGGAAG		genome	216 bp	
UBQ10-F	Sense	TGCATCTCGTTCTCCGATTG		Cassava		
UBQ10-R	Antisense	GCGAAGATCAGTCGTTGTTG		genome	107 bp	

CP* Coat protein
UTR** Untranslated region

3.3.5 Selection of species specific restriction endonuclease for RFLP analysis

Species-specific restriction endonucleases were selected using nucleotide sequences 752-785 bp length corresponding to CP region amplified by the degenerate primer designed in this study **(Table 8).** Computer software package Vector NTI (Informax Inc., Bethesda, MD, USA) was used to search for endonucleases that cut few distinguishable sites/fragments within the amplified CBSV and CBSUV sequences. Two species-specific endonucleases *Eco*RI and *Hind*III were identified for specific identification of CBSV and CBSUV both in single and mixed infection (**Figure 12**). To ascertain whether the selected endonucleases will work efficiently for all CBSVs isolates, all CBSV and CBSUV sequences available in the database were retrieved and trimmed to 752-785bp corresponding to the size of the designed degenerate primers, and used to check the specificity of the selected endonucleases in discriminating the two CBSV species using computer-based program Vector NTI (**Table 7**).

For RFLP analysis, RT-PCR amplicons were digested by *Eco*R1 and *Hind*III and incubated at 37^0C for 1.5 hours as per manufacturer's instructions (MBI, Fermentas, Vilnius, Lithuania). The restriction digest was run on a 2% agarose gel for 100V/1hr, stained with ethidium bromide (2 µl of 10 mg/ml) and visualized under UV light using UVP BioDoc-It imaging system. Sizes of the restricted fragments were determined by 1KB Plus ladder. The RT-PCR/RFLP method was performed at least two times for each CBSV and CBSUV samples.

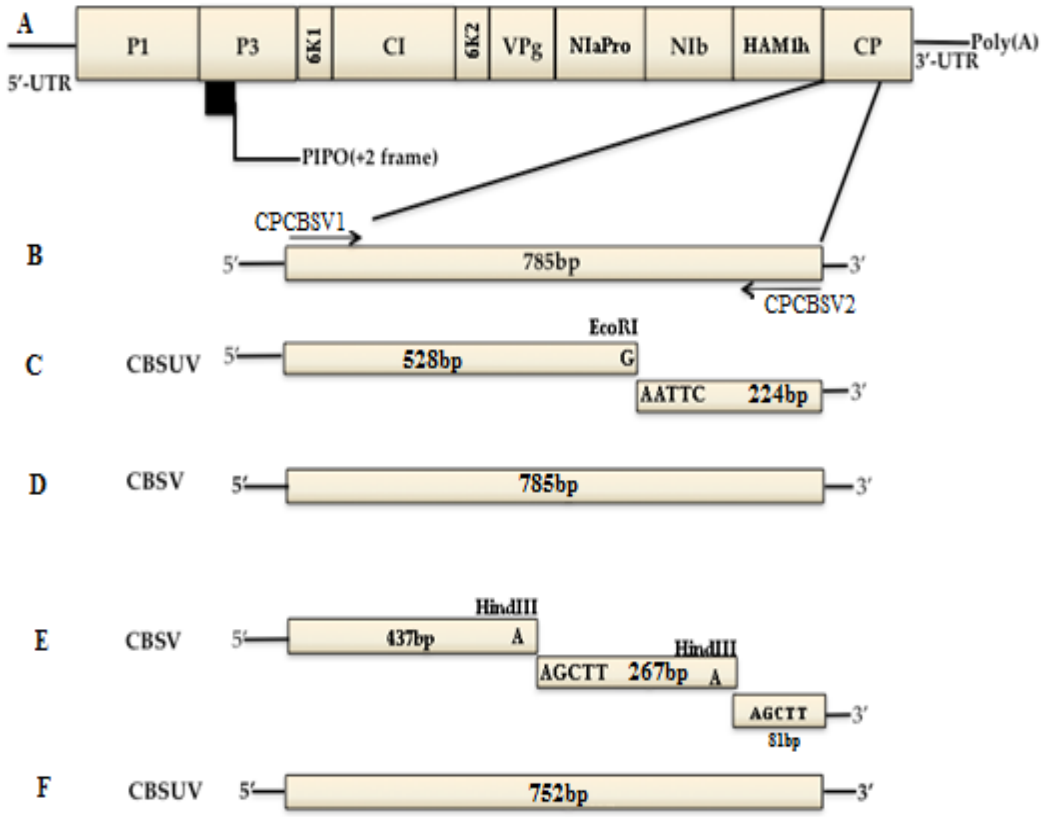


Figure 12: A and B: Schematic representation of the CBSVs genome showing the amplified region in the coat protein region by the primer pair CPCBSV1/2. **C** and **D:** Computer predicted restriction sites by *Eco*RI on CBSUV (525, 224bp) and CBSV (785bp), **E** and **F:** predicted restriction sites by *Hind*III on CBSV (437, 267& 81bp) and CBSUV (752bp). The computer programme vector NTI suite8 (Informax, Wisconsin, USA) was used to predict restriction sites using selected endonucleases.

3.3.6 Specificity of endonucleases in discrimination of CBSVs

Representative RFLP patterns for CBSV and CBSUV were gel-eluted, purified and sequenced at BecA/ILRI, Nairobi, Kenya. The identities of the virus species were achieved through BLASTn search in the GenBank.

3.3.7 Validation of RT-PCR/RFLP assay and comparison with other assay

RT-PCR/RFLP assay was used to screen for CBSV and CBSUV from the leaf samples collected from different countries as described in section 2.1 above. Two other separate assays including; Abarshi et al. (2010) and Mbanzibwa et al (2011) were conducted on the collected leaf samples using the same cDNA synthesized by oligo (dT) 25. Positive and negative controls used are described in section 3.3.3. To avoid false-negative results all samples used were tested by amplifying the control reference genes Ubiquitin10 (UBQ10) and PVD2 (Moreno et al., 2011) using primers UBQ10-F/R and PVD2-F/R respectively (**Table 8**).

3.4 Results

3.4.1 Sample collections and DNA extraction

The integrity of the preserved leaf sample was excellent after a month dry storage. Both greenish colour and vein clearing symptoms could still be seen clearly (**Figure 11D**). High quality RNA was extracted from one-month old dry stored cassava leaf samples using modified CTAB extraction protocol (**Figure 13**). The extracted RNA was used successfully in all downstream operations including RT-PCR, cloning and RFLP. Gel electrophoresis showed clear RNA bands from dry leaves, which were comparable to those obtained from fresh leaves using the same RNA extraction protocol (**Figure 13A&B**). Comparison of extracted RNA between dry and fresh leaves showed no significant difference ($P<0.05$) in RNA concentrations using the modified CTAB protocol (**Table 9**). Average RNA concentrations recovered from 50 mg of fresh and dry leaves were 2.5 and 2.1 µg/µl respectively (**Table 9**). The sensitivity of the developed

RT-PCR/RFLP was assessed by establishing detection limits of the RFLP assay in 10 fold (10^{-1} to 10^{-6}) serial dilutions of total RNA. The assay detected CBSVs in field samples up to dilution of 10^{-4}, and sensitivity decrease with increased dilution to 10^{-5} (Data not shown).

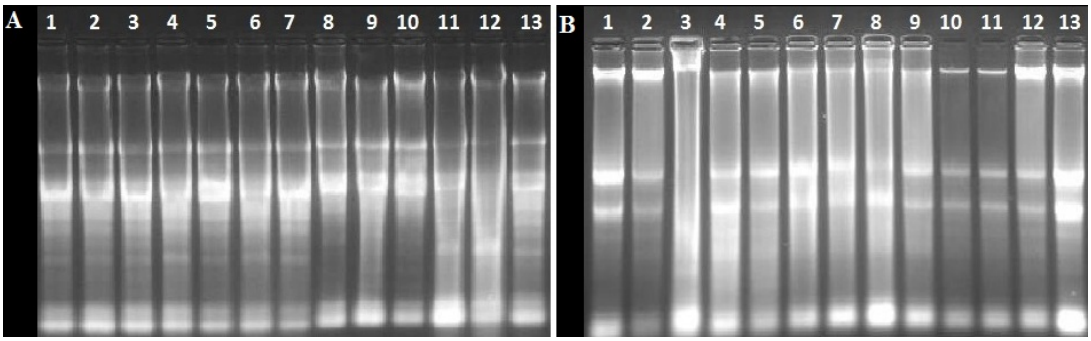


Figure 13: Agarose gel electrophoresis of RNA extracted from cassava leaves. A: RNA isolated by the modified 2% CTAB extraction protocol from fresh cassava leaves; B: RNA isolated from dry cassava leaves by the same extraction method.

Table 9: RNA concentration for fresh and dry leaf samples extracted using the modified CTAB protocol

Sample ID	Nucleic Acid Conc. (ng/µl)	A260	A280	260/280	260/230
Fresh leaf					
1	2177.0	54.425	26.886	2.02	2.19
2	2773.2	69.331	33.323	2.08	2.16
3	1550.1	38.753	18.819	2.06	2.09
4	2268.1	56.702	28.285	2.00	2.02
5	2884.3	72.107	34.976	2.06	2.14
6	1666.0	41.651	20.381	2.04	2.11
7	2642.5	66.063	32.056	2.06	2.07
8	3723.0	93.074	45.831	2.03	2.06
9	1502.0	37.550	18.890	1.99	1.92
10	3966.2	99.156	51.483	1.93	1.82
11	2403.9	60.098	30.235	1.99	1.95
12	1492.7	37.318	18.083	2.06	2.14
13	3473.5	86.836	42.113	2.06	2.09
14	3946.9	98.672	50.103	1.97	1.89
15	2064.8	51.619	25.245	2.04	1.99

Dry leaf					
1	2750.2	68.755	33.082	2.08	2.16
2	2813.1	70.327	34.673	2.03	1.82
3	3125.5	78.136	38.348	2.04	1.81
4	1772.0	44.300	21.844	2.03	1.86
5	1725.3	43.133	21.122	2.04	1.98
6	2569.8	64.245	31.567	2.04	1.84
7	2678.1	66.952	32.695	2.05	1.89
8	1630.1	40.752	20.597	1.98	1.62
9	2330.7	58.267	28.672	2.03	1.84
10	1238.0	30.949	14.944	2.07	2.11
11	1516.9	37.922	18.496	2.05	1.90
12	1847.1	46.177	23.818	1.94	1.56
13	1316.8	26.336	12.481	2.11	2.16
14	3477.4	69.548	33.061	2.10	2.16
15	1342.3	33.557	16.444	2.04	1.87

Mean of fresh leaf = 2,569 ng/µl
Mean of dry leaf = 2,142 ng/µl
PV = 0.157, $\alpha = 0.05$, LSD = 601.2

3.4.2 RFLP analysis

Computer-based RFLP analysis generated two fragments (528 and 224 nt) when CBSUV was digested with *Eco*RI from the 785 nucleotide (nt) sequence corresponding to the targeted coat protein region **(Table 7).** When a similar sequence for CBSV (785nt) was digested with *Eco*RI the fragment remained uncut **(Table 7)** for all CBSV sequences. In contrast, computer analysis of a CBSV (785 nt) sequence cut with *Hind*III generated three fragments (437, 267 and 81 nt), and for CBSUV (752nt) sequence the fragment remained uncut **(Table 7).** In the case of mixed infection of CBSV and CBSUV, digestion with *Eco*RI and *Hind*III generated three (785, 525 and 224 bp) and four (785, 437, 267 and 81 bp) fragments, respectively **(Table 7).**

Digestion of PCR amplicons from known CBSV, CBSUV and mixed infected samples **(Figure 14A)** by *Eco*R1 and *Hind*III restriction endonucleases **(Figure 14B&C)** showed conformity to the restriction patterns generated by the computer analysis in **figure 3A-F.** Digestion by *Eco*R1 the PCR products remained uncut **(Figure 14B lane 1-5**) for CBSV-

infected samples while for CBSUV-infected samples two fragments of 528 and 224 bp were observed **(Figure 14B, lane 6-10).** Digestion of PCR amplicons from co-infected samples with *Eco*RI resulted in three fragments, 785, 528 and 224 bp, which is consistent with co-infection of CBSV and CBSUV **(Figure 14 B lane 11-15).** Similarly, *Hind*III digestion generated the expected fragments of 437, 267 and 81 bp for CBSV and 752 bp for CBSUV in a singly infected sample. Four fragments, 785, 437, 267 and 81bp were observed from the digestion of PCR products from co-infected samples using *Hind*III (**Figure 14 C lane 11-15**).

Reliability of RT-PCR/RFLP results was compared with results generated using primers CBDDF2/CBSDDR (Mbanzibwa *et al.*, 2011) **(Figure 14D).** Sample number 1-5 produced a 344 bp fragment typical of CBSV; sample number 6-10 produced 438–440 bp indicating CBSUV infection, while sample numbers 11-15 had two bands at the position of CBSV and CBSUV, indicating a mixed infection of the two virus species.

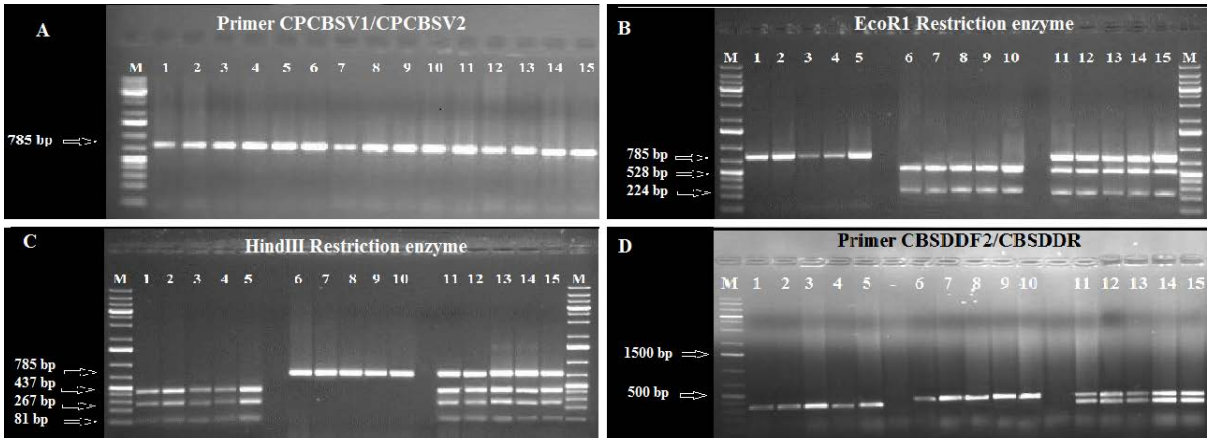


Figure 14A: RT-PCR product of CBSVs isolates universally amplified by the degenerate primer CPCBSV1/2**. B and C:** subsequent digestion by *Eco*Rl and *Hind*III endonucleases respectively, **D:** RT-PCR amplification of the same samples with specie specific primer CBSDDF2/CBSDDR (Mbanzibwa *et al*., 2011). Reaction products were separated by 2% agarose gel electrophoresis and visualized under UV light

3.4.3 Comparison of RT-PCR/RFLP with other tools in CBSVs detection

In order to validate the reliability of RT-PCR/RFLP tool in detection and discrimination of CBSVs, a total of 72 cassava leaf samples with CBSD-like symptoms from Mozambique (24), Tanzania (24) and Uganda (24) were analyzed using the RT-PCR/RFLP, and compared for simultaneous detection of CBSV and CBSUV with RT-PCR assays from Mbanzibwa *et al.* (2011) where primers CBSDDF2/CBSDDR were used, and a RT-PCR assay using three primer combinations (CBSVF2/ CBSVR7/CBSVR8) (Abarshi *et al.* (2010). Results showed that CBSVs were both detected in single and in mixed infections **(Figure 15A-D**) in the tested samples. CBSV was more readily detected in single infections than CBSUV in all the 3 assays used **(Figure 15A-D**). Results from amplification of the reference genes (PVD2 an UBQ10), showed a clear amplification of all the tested samples except the water control which was negative (figure not shown) demonstrating that the cDNA was from cassava genome.

In the RFLP assay using *Eco*RI and *Hind*III restriction enzymes, CBSV was respectively detected in 54% and 50 % samples from Mozambique, 16.7% each from Tanzania and 20.8% each from Uganda, while CBSUV was respectively detected in 4.2% each from Mozambique, 45.8% and 37.5% from Tanzania and 17.7% each from Uganda samples **(Figure 15A&B).** On the other hand, dual infection of CBSV and CBSUV using *Eco*RI and *Hind*III was respectively detected in 8.3% and 12.5% samples from Mozambique, 37.5% and 45.8% samples from Tanzania and 25% and 29.2% samples from Uganda **(Figure 15A&B).**

Our results agree with those obtained using Mbanzibwa *et al.,* (2011) diagnostic RT-PCR assay. Of the 24 samples analyzed from Mozambique using Mbanzibwa *et al.,* (2011) assay, 62.5% were singly infected by CBSV, 4.2% by CBSUV. In Tanzania samples, CBSV and CBUSV were detected at frequency of 20.8% and 45.8%, respectively, while 33.3% of leaf samples were dually infected by CBSV and CBSUV. Detection of samples from Uganda showed 50% infection by CBSV and 4.2% by CBSUV and 8.3% dually infected by CBSV and CBSUV **(Figure 15C).**

However, when the same samples were analysed by the Abarshi *et al.,* (2010) RT-PCR assay, the number of samples detected positive for CBSV and CBSUV decreased **(Figure 15D).** For instance, in Mozambique samples the only virus detected by Abarshi *et al.,* (2010) was CBSV in 17.7% of samples. Neither CBSUV nor co-infection of CBSV and CBSUV was detected in the samples using Abarshi *et al.,* (2010) RT-PCR assay **(Figure 15D).** Of the Tanzanian samples, 12.5% were CBSV-infected, while 25% were infected with CBSUV, and no leaf samples were positive for dual infection of CBSV and CBSUV, while for the Ugandan samples only CBSV was detected in 50% of leaf samples, and neither CBSUV nor dual infection of CBSV and CBSUV were detected using Abarshi *et al.,* (2010) RT-PCR assay **(Figure 15D).**

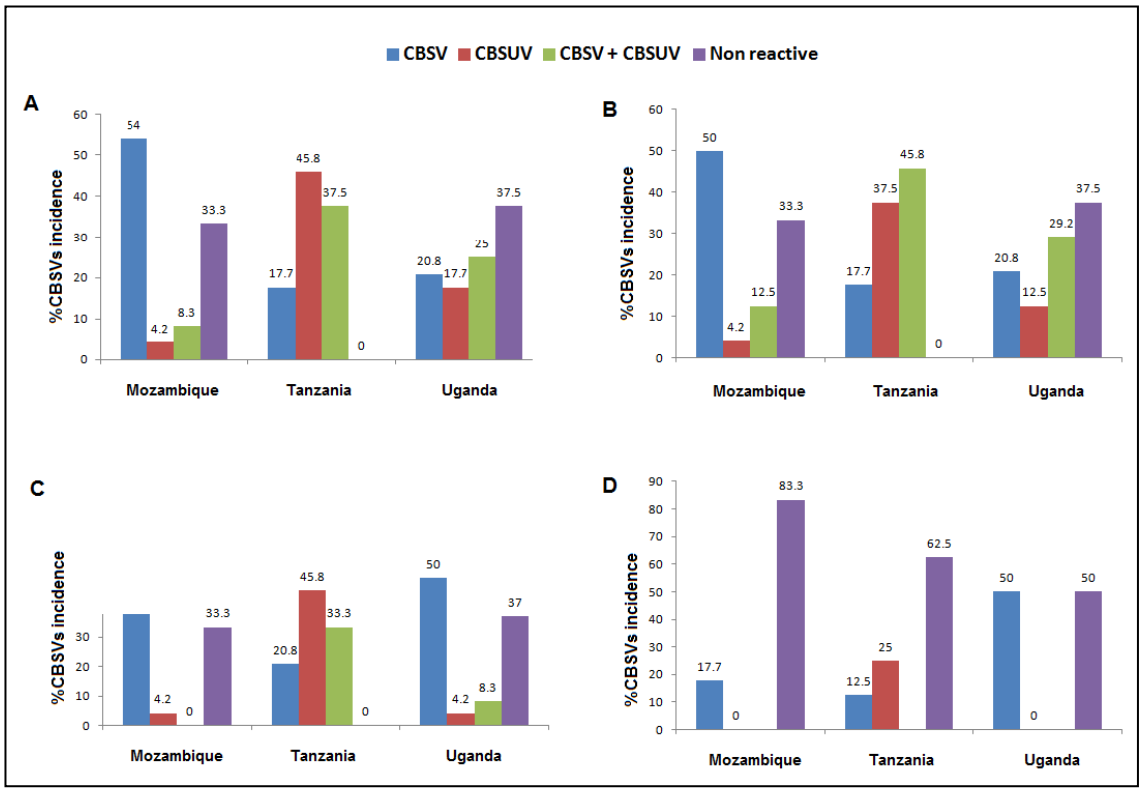


Figure 15: Comparison of the proportions detection of CBSVs in single and mixed infection from the field samples from Mozambique, Tanzania and Uganda. **A:** Digestion of CPCBSV1/2 amplified PCR product by EcoRI, **B:** Digestion of CPCBSV1/2 amplified PCR product by HindIII, **C:** RT-PCR detection by specie specific primer CBSDDF2/CBSDDR (Mbwanzibwa et al., 2011) and **D:** RT-PCR detection by Abarshi et al., (2010) primers CBSVF2/ CBSVR7/CBSVR8.

The overall detection for the 72 samples from Mozambique, Tanzania and Uganda showed CBSVs were equally detected (76.4%) by RT-PCR/RFLP and Mbanzibwa *et al.* (2011) assays, while the Abarshi *et al.* (2010) only detected 34.7% **(Table 10).** Country wide, CBSVs were detected in 66.7% of samples tested from Mozambique, 100% from Tanzania and 62.5% from Uganda by both our RFLP and (Mbanzibwa *et al.*, 2011) detection assays **(Table 10).** In contrast, the Abarshi *et al.*, (2010) only detected the CBSVs in 16.7%, 37.55% and 50% from Mozambique, Tanzania and Uganda samples, respectively **(Table 10).**

Table 10: Overall detection and comparison of the different tools used in this study in detecting CBSVs in field infected samples

Country	CPCBSV1/CPCBSV2	CBSDDF2/CBSDDR	CBSVF2/ CBSVR7/CBSVR8
Mozambique	16 (66.7%)	16 (66.7%)	4 (16.7%)
Tanzania	24 (100%)	24 (100%)	9 (37.5)
Uganda	15 (62.5%)	15 (62.5%)	12 (50%)
Total	55 (74.3%)	55 (74.3%)	25 (34.7)

3.5 Discussion

In this study I have shown that CBSVs can be detected and discriminated by RT-PCR/RFLP. The patterns obtained with the RFLP digestions using *Eco*RI and *Hind*III were consistent with the computer-based digestions of published sequences for CBSV and CBSUV. The RT-PCR/RFLP assay detected and discriminated CBSV and CBSUV from the field samples. However that, results of digestion with *Eco*RI and *Hind*III were not consistent could indicate more variability among the *Cassava brown streak viruses* (CBSV and CBSUV) than could be detected by the RT-PCR based assays. This was confirmed with sequencing (data not presented).

The results obtained by the RT-PCR RFLP assay in this study compared well with those by the Mbanzimwa et al. (2011) primers for the overall analysis of the CBSVs in the three countries. However, for detailed analysis of single vs mixed infections, some mixed infections were detected with the RT-PCR/RFLP assay in samples that were previously detected as single infections by the Mbanzibwa et al. (2011) assay. Present

results suggest that RT-PCR/RFLP assay is more sensitive in detecting mixed infections than the Mbanzibwa et al. (2011) assay. For instance, using samples collected from Mozambique, CBSUV was detected only by the RFLP assay, while the PCR-based assays by Abarshi et al. (2010) and Mbanzibwa et al. (2011) could not. The assay can therefore be used to compliment other diagnostic assays in detecting CBSV and CBSUV. Results from this study using the RT-PCR/RFLP assay indicated that CBSV and CBSUV are widely distributed in Tanzania and Uganda in single and mixed infections as reported by Mbanzibwa *et al*. (2011); Adam *et al*. (2012) and Winter *et al*. (2010).

Availability of more CBSV and CBSUV sequences in the GenBank greatly enhanced the designing of the degenerate primers (CPCBSV1/2) in this study. The forward primer (CPCBSV1) was designed 165 bp in the N-terminal, where the two viruses differ (Winter *et al.,* 2010) by 33 bp (785 and 752bp), which was evident in the size of the PCR products obtained. The reverse primer (CPCBSV2) was designed from the C-terminal of the coat protein resulting in amplification of mainly conserved core region of the CP. The new degenerate primers detected similar proportions of sample with CBSVs as obtained with the Mbazibwa et al. (2011) multiplex primers. However, it is not clear why the Abarshi et al. (2010) multiplex primers produced many no amplification results.

I report on successful amplification of CBSVs from dried herbarium leaf samples and demonstrated the utility of the CTAB protocol for the diagnosis of cassava RNA viruses. Abarshi *et al.* (2012) also extracted RNA from dried herbarium cassava leaves using CTAB protocol, and successfully amplified CBSVs. In this study I further demonstrate a stepwise procedure for collecting and storing dry leaf samples in the field. The dry leaf samples retained the green color and visible vein chlorosis of CBSD symptoms after a

month of storage. Extraction of good quality RNA was successfully achieved from the dry stored cassava leaf samples and used for other downstream assays, such as RT-PCR/RFLP with comparable results with fresh leaf samples.

3.6 Conclusion

PCR/RFLPs have successfully been used for the detection and differentiation of cassava mosaic DNA begomoviruses (CMB) infecting cassava (Okao-Okuja *et al.,* 2004; Sseruwagi *et al.,* 2004; Boraha and Dasgupta, 2012). The development of a combined RT-PCR/RFLP assay is a valuable tool where cloning and sequencing are limited and costly.

This study has shown that RT-PCR/RFLP assay successfully detected and discriminated CBSV and CBSUV in single and mixed infected cassava field samples. Use of dry cassava leaf samples produced high quality RNA for RT-PCR/RFLP. The RT-PCR/RFLP technique demonstrated that CBSVs could reliably be diagnosed in the laboratories within a short period of time without a requirement for expensive advanced equipment for sequencing. The technique is suitable in screening for virus disease resistance, diagnostic studies, checking movement of diseased planting materials, and virus indexing.

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CHAPTER FOUR

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Cassava mosaic and Cassava brown streak diseases have continued to be the most important diseases lowering cassava productivity in the whole of sub-Saharan Africa. The two diseases are caused by viral infection and occur in single and in confection. CMD is caused by at least seven species and variants of genus begomovirus, family *Geminiviridae* (Fauquet *et al.,* 2008) and CBSD is caused by two species of CBSVs (Mbanzibwa *et al.*, 2009a).

Despite tremendous efforts that have been made on developing a reliable diagnostic assays for these viruses using biological, serological and molecular techniques, a comprehensive assay(s) that can simultaneously detect both viruses (CMB and CBSV) species and their variants have remained a challenge and hence management.

In this study a single-tube duplex and multiplex PCR for simultaneous detection of four cassava mosaic begomovirus species (ACMV, EACMCV, EACMMV and EACMZV) and RT-PCR based RFLP for identification and differentiation of two CBSVs species (CBSV and CBSUV) were developed, optimized and utilized to detect and determine the diversity of CMBs and CBSV in cassava field samples.

Four species-specific primers were developed from the DNA-A component of CMGs and optimized for single tube and multiplex PCR. The multiplex PCR was used to index 172 field collected cassava samples from Tanzania, Kenya, Rwanda, Malawi and

Mozambique. All four specific primer pairs specifically detected the expected begomoviruses.

The specificity of the developed assay was high and the sensitivity was also high, the four begomoviruses were detected in a serially diluted DNA samples up to 10^{-4}. This is the first study that has revealed the diversity of CMBs in Rwanda, Malawi and Mozambique with the occurrence of both EACMCV and EACMZV in single and mixed infection.

A single degenerate primer pair (CPCBSV1/2) targeting the conserved region in the coat protein gene of the two CBSVs species was developed and, combined with RFLP using two species-specific endonucleases *Eco*RI and *Hind*III for detection and differentiation of CBSV and CBSUV. The RT-PCR/RFLP assay was used to detect CBSVs species in 72 field-collected cassava samples with CBSD-like symptoms from Tanzania, Uganda and Mozambique. CBSV and CBSUV were successfully detected in single and mixed infection. Comparison with other tools shows that developed RT-PCR/RFLP detected more viruses species from field collected samples than Abarshi *et al.,* (2010) multiplex assay and produced comparable results with Mbanzibwa *et al.,(* 2011 PCR assay. The RT-PCR/RFLP assay for detection and discrimination of CBSVs developed in this study compliment other assays for detection of CBSVs.

The other development made in this study toward developing cost-effective diagnostic assays is modification of sample collection and nucleic acid extraction protocols. The DNA extraction protocols (Dellapotra *et al*., 1983) and RNA extraction by CTAB (Chang *et al*., 1993) were slightly modified to use dry leaf samples without liquid nitrogen. The resultant DNA and RNA were of high quality and suitable for downstream applications

PCR and RT-PCR/ RFLP, respectively. The improved sample collection significantly simplified the diagnosis of both CMBs and CBSVs. Exclusion of liquid nitrogen save costs and the need of unavailable liquid nitrogen in some countries. Again use of dry stored leaves similarly minimized losses that are associated with deterioration during transporting samples from remotely sampling districts to urban located central diagnostic laboratories.

In conclusion, the present study using developed assays for CMBs and CBSVs, the occurrence and diversity of four CMBs and 2 CBSV species were revealed in Tanzania, Uganda Rwanda, Malawi and Mozambique. Though the occurrence of 4 CMBs and 2 CBSVs was expected in Tanzania and Uganda, the detection of CBSUV in Mozambique is a recent discovery. This confirms the first report of CBSUV occurrence in Mozambique (Amissie *et al.,* Unpublished report).

Recommendations

The present study has open more areas for further research in order to further improve the diagnostic assays to form comprehensive diagnosis of CMBs and CBSVs. The areas for more research include:

1. Further refinement of diagnostic tools is needed to cover other CMBs species that are not detected with current available multiplex tools in order to have a cost effective tool for comprehensive diagnosis of CMBs causing CMD

2. More studies/tools developed to establish if there are more virus species and/or variants associated with cassava brown streak disease

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