# EPIDEMIOLOGICAL ASPECTS OF CASSAVA BROWN STREAK DISEASE IN FIELD GROWN CASSAVA IN COASTAL REGIONS OF TANZANIA

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**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.**

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## ABSTRACT

The study aimed at determining the spatial and temporal spread of cassava brown streak disease (CBSD) in field grown cassava in Chambezi, coastal Tanzania and identifying the alternative hosts of *Cassava brown streak virus viruses (CBSVs)*. In determining the spatial and temporal spread of CBSD three treatments were used in the study and a no disease plot served as a control. Data were collected on CBSD severity, incidence and whitefly counts and analysed using SPSS for analysis of variance. Graphs for the temporal disease spread were established to explain the temporal disease spread and examined. Based on the shapes suggest that CBSD progressively increased with time, limited effects of blocking and treatments on foliar and stem incidence of CBSD was observed. The effect of time was highly significant on foliar and stem incidence ($P < 0.005$). In addition, the effect of blocking, treatments and time (MAP) was highly significant on whitefly population ($P < 0.005$). The study has shown that susceptible CBSD cassava cultivars become infected though CBSVs infected cassava plant(s) planted in the farm. In determining the alternative hosts of CBSD leaf samples were collected from shrubs and herbs with-virus like symptoms in the Coast, Dar es Salaam and Tanga Regions. Detection of CBSVs was done where samples from *Annona senegalensis* Pers. tested positive to CBSV whereas UCBSV was detected in *Solanum incanum* L. *Psorospermum febrifugum* Spach.var tested positive for the two viruses (CBSV and UCBSV) suggesting coinfections. This new findings sheds light on the origin of this viruses that was first reported from East Africa at Amani in Tanga Region. It further suggests that cleaning of new stock from virus might not offer an effective solution to CBSD management in areas where alternative host plants are rampant.

## DECLARATION

I, Catherine Bura Gwandu, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Catherine Bura Gwandu Date

(MSc. Candidate)

The above declaration is confirmed:

_____ _____

Dr. Gration Rwegasira Date

(Supervisor)

## COPYRIGHT

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## DEDICATION

To my husband Dr. Paschal Nkii and my lovely twins Brown and Brian who stayed lonely in my absence at home but were patient. Also my maid Zaitun Ramadhani who took care of my children during my absence and showed love to my children throughout my study period.

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# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background Information

Cassava (*Manihot esculenta* Crantz) is a shrubby perennial plant of the family Euphorbiaceae that typically grows from one to three meters (3-10 feet) in height (Thresh *et al.,* 1998). Among the 28 known species in the *Euphorbiaceae* family, cassava is the only edible crop and its tuberous roots are a good source of carbohydrates (Katz and Weaver, 2003). The crop is a key staple in several African, South American and Asian countries, and has the highest production potential calories per hectare per day among tropical crops (De Bruign and Fresco, 1989). In most African countries, cassava is grown for food by small scale farmers on subsistence basis and it constitutes an important source of income in rural and often marginal areas, especially for women (Legg *et al.,* 1999).

As raw material, cassava can be processed into a wide variety of products for food and feed such as starch, flour, ethanol, glucose and others. The leaves which are rich in proteins, vitamin A and other nutrients are consumed in some communities to supplement the low protein content of the roots (FAO, 2006). Although cassava is poor in nutritional elements, a recent study has indicated that root qualities would be improved through transgenic biofortification (Abhary *et al.,* 2011). Although cassava is a very hardy plant, which tolerates drought better than most crops, and can grow well in very poor and acidic soils (Katz and Weaver 2003), its yield, especially in Africa is very low (Bender, 2005) estimated losses due to CBSD is between 35-70 million US$ in Tanzania (Calculated at a price of 100 US$/Mt fresh cassava) (Ndunguru *et al.,* 2010).

The productivity of the crop is hampered by a variety of abiotic (poor soils, drought and poor crop husbandry) and biotic (low yielding varieties, pests and diseases) factors.

Of the biotic factors, viral diseases are the key limitation to cassava production reported to contribute to enormous yield losses (Thresh *et al.,* 1998). Two viral diseases namely Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD) are known to affect cassava productivity in the vast of sub Saharan Africa (Alicai *et al.,* 2007). CMD is caused by the *Cassava mosaic virus* belonging to the genus *Begomovirus* and family *Geminiviridae* whilst CBSD is caused by two viruses, the C*assava brown streak virus* (CBSV) and *Cassava brown streak Ugandan virus* (CBSUV). Both CBSV and CBSUV are members of the genus *Ipomovirus* in the family *Potyviridae*.

Cassava brown streak disease (CBSD) was first reported to occur in Tanganyika now Tanzania in 1936 at the foothills of Usambara Mountains (Story, 1936). The epidemiology of CBSVs is poorly elucidated partly because its source of infection that could not immediately be established. Information also regarding its rate of spread within and between cassava fields is scanty. The disease was historically known to be endemic to the coastal lowlands of Eastern Africa at less than 500 masl, until in 2007 and 2009 when the disease was reported at higher altitude above 1200 meters above sea level (masl) in Uganda and Tanzania respectively (Alicai *et al.,* 2007; Rwegasira, 2009). The disease is currently widespread in the Eastern, Central and Southern Africa region in such countries as Burundi, DRC, Kenya, Malawi, Mozambique, Rwanda, Tanzania, Uganda and Zambia (Alicai *et al.,* 2007). Yield losses associated with CBSD infection was previously estimated at 70% per plant (Hillocks *et al*., 2001), but recent records indicated up to 100% yield losses in susceptible cultivars (Mbanzibwa *et al.,* 2011; Rwegasira, 2009). CBSV has been included in the list of top six dangerous pathogens of crops around the world (Pennis, 2010).

**1.2 Justification**

Cassava is one among the vital good for Africa due to that fact that it can yield relatively low even in poor soils with limited inputs. However, its productivity is hampered by a

series of biotic stresses particularly the viral diseases namely CMD and CBSD. Recent efforts geared to understand CBSD have focused greatly on characterising the molecular variability and transmission of the causative viruses (Mbanzibwa *et al.,* 2011; Rwegasira *et al*., 2011). Whiteflies (*Bemisia tabaci*) were reported to transmit CBSV, albeit at a very low efficiency (Maruthi *et al.*, 2005). The spiralling whitefly (*Aleurodicus dispersus*) has also been demonstrated to transmit the viruses that cause CBSD (Mware *et al.,* 2009).

However, knowledge is lacking on the spatial and temporal spread of CBSD in the field. Furthermore, it has long been suspected that alternative host plants could serve as reservoirs for CBSV and CBSUV and may be sources of inoculums for new infections to susceptible cultivars when field grown even if farmers had planted CBSV-free planting materials (Hillocks and Jennings, 2003). Unfortunately, wild plants harbouring CBSV and CBSUV have not been identified. Recently, *Manihot glaziovii*, which is a close relative to cassava, was shown to be infected with CBSV (Mbanzibwa *et al*., 2011). Not even a single alternative host has been found for CBSUV. Moreover, lacking reports on the availability and the possible sequence diversity for both CBSV and CBSUV makes it imperative to explore the alternative host plants. Aavailability of sequences of isolates from wild species could shed some light on the evolution of CBSV and CBSUV and thus improve our understanding of the adaptation of these viruses to cassava as their new host. This is an important aspect especially in predicting the potential of CBSV and CBSUV isolates to evolve into new strains or even distinct viruses. A clear understanding of the epidemiology of CBSD is a key to developing informed management strategies. The proposed study aims to determine the spreading pattern of CBSD in cassava fields and identify alternative hosts for CBSV and CBSUV in the coastal regions of Tanzania.

## 1.3 Objectives

### 1.3.1 Overall objective

To understand the nature and contributing factors to the occurrence of CBSD in field grown cassava in the Coast areas in Tanzania.

### 1.3.2 Specific objectives

i. To examine the spatial and temporal spread of CBSD in selected CBSV-free cultivars in coastal Tanzania

ii. To identify the alternative host plants for CBSV in coastal Tanzania

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Cassava Brown Streak Disease

Cassava brown streak disease is a viral disease, which was first reported to be endemic in cassava growing areas in coastal East Africa at altitudes less than 1000 masl (Storey, 1936; Nichols, 1950). CBSD symptoms vary depending on the part of plant affected, cultivar, crop age, and weather conditions (Hillocks, 1999; Nichols, 1950). On leaves, the disease appears as a feathery chlorosis on either side of the small veins (Nichols, 1950). Characteristic CBSD foliar symptoms normally occur only on mature leaves; young expanding leaves are usually symptomless (Bock, 1994). Economic damage occurs on the tuberous roots as yellow/brown, corky necrosis in the starch-bearing tissues, and radial root constriction, which occurs at very severe infections in sensitive cultivars (Hillocks, 2003). Necrosis begins as discrete areas, but in very susceptible cultivars. It may affect most of the root, rendering them unfit for human consumption (Hillocks and Jennings, 2003).

### 2.2 Genetic Diversity of CBSV and CBSUV

Genetic variability of CBSV and CBSUV for isolates infecting cassava has been studied widely (Mbanzibwa *et al.*, 2009; Mbanzibwa *et al.*, 2010b; Winter *et al.*, 2010; Monger *et al*., 2010). There are 12 complete genomes in the GenBank and over 70 complete coat protein sequences and several partial sequences of different genes of CBSV and CBSUV. However, there are no sequences of the isolates from wild plants. A few partial sequences of CBSV isolates from *M. glaziovii* sub-clustered in the main clade of CBSV suggesting a continuous evolution and therefore genetic distinctness (Mbanzibwa *et al.,* 2011).

## 2.3 Transmission of CBSV

First demonstration of CBSV transmission was done mechanically (Lister, 1959) and later on by whiteflies (Maruthi *et al.,* 2005; Mware *et al*., 2009). Existing reports indicate that two viruses are the causal agents of CBSD in East Africa including: *Cassava brown streak virus* (CBSV) and *Cassava brown streak Uganda virus* (CBSUV) (Mbanzibwa *et al.,* 2009; Monger *et al.,* 2010; Winter *et al.,* 2010).

Rwegasira (2009) demonstrated that CBSV was graft transmissible and that cuttings from infected plants invariably gave rise to plants showing symptoms of CBSD. As cassava is usually propagated by stem cuttings, the disease is readily introduced into newly planted areas through the use of infected planting material. In the most sensitive varieties, severe symptoms develop when the disease is established at early stage (Nichols, 1950). Earlier report pointed out that CBSV was insect-transmitted and that the most probable vector was the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Storey, 1939). Several attempt to transmit CBSV by vectors, including aphids (*Myzus percicae Sulz.*) (Lennon *et al.,* 1986) and *B. tabaci* (Bock, 1994) were fruitless. However, the virus was successfully transmitted albeit at low efficiency of 22% at elevated temperatures above $28^0$C (Maruthi *et al.,* 2005). Spiraling whitefly-like pest (*Aleurodicus disperses*) (Russell) has also been suspected to transmit CBSV viruses (Mware *et al*., 2010) with limited proofs. It is worthy noting that at the time CBSV was shown to be transmitted by *B. tabaci*, the CBSUV isolates remained undiscovered and the CBSV10 vs. CBSV11 coat protein primers used to confirm the transmission could not distinguish between the two viruses.

## 2.4 Alternative Hosts for CBSD

*Petunia hybrid* (Yolanda Vanveen) was confirmed the most susceptible alternative host plant to CBSV (Mware *et al.,* 2010). Subsequent studies reported successful transmission

of CBSV in *Nicotiana benthamiana* and *Nicotiana debneyi* (Bock, 1994). CBSV was also recently detected in *Manihot glaziovii*, a close relative of cassava (Mbanzibwa *et al.*, 2011). Mware *et al.* (2010) have noted a large number of plants that are fed on by whiteflies and thus could be potential alternative hosts to CBSV. Natural hosts of CBSV are not known hence the need for research to generate information upon which formulation of effective CBSD management strategies would be based.

**2.5 Diagnosis and Detection of CBSV**

Manifestation of CBSD symptoms on sensitive cultivars has often been used in diagnosing for CBSV infections. The disease symptoms are expressed on foliar, stem and storage roots. Foliar symptoms of CBSD appear like feathery chlorosis on either side of the small veins. Characteristic CBSD foliar symptoms normally occur only on mature leaves and the young expanding leaves are often symptomless (Bock, 1994). In stems, necrotic spots are often seen which enlarges in to nectotic blotches with subsequent die-back starting from the tope green portion of the stem. Tuberous roots are yellow/brown, corky necrosis in the starch-bearing tissues, and radial root constriction may occur in very sensitive cultivars (Hillocks, 2003).

Symptomatic leaves are usually sampled for the reverse transcriptase-polymarase chain reaction (RT-PCR) based CBSV detection due to the fact that reliance on symptoms for diagnosis of CBSD is not reliable because some cultivars may phenotypically remain symptomless despite being infected (Abarshi *et al*., 2010). Thus, several RT-PCR diagnostic protocols for CBSV have been developed for diagnosis of the disease (Abarshi *et al*., 2010; Monger *et al*., 2001, Rwegasira, 2009; Rwegasira *et al.*, 2010).

## 2.6 Management of CBSD

Storey (1936) and Nichols (1950) recommend selection and use of disease free cuttings for planting material. However, both authors pointed out that this was not easy due to the fact that in susceptible cultivars, the disease free plants would probably be too few to provide sufficient planting materials. Moreover, CBSD diagnosis is not always straightforward and apparently symptomless plants could be latently infected (Rwegasira and Rey, 2012). Therefore, the use of resistance varieties as a means of controlling CBSD was recommended (Jennings, 1960).

A Natural Resources Institute (NRI, UK) review mission carried out in 2005 concluded that whilst it is no longer possible to eradicate CBSD, it was important to take common action by all stakeholders to restrict the movement of cassava cuttings through the open quarantine system (Bock, 1994). It was further advised that only virus-tested tissue culture materials be used for inter country cassava germplasm movement.

# CHAPTER THREE

## 3.0 GENERAL MATERIALS AND METHODS

### 3.1 Analysis of Spatial and Temporal Spread of Cassava Brown Streak Disease in Coastal Tanzania

The study was conducted for one growing season at one experimental site Chambezi, in Coast Region. It was two year study and laboratory activities were carried out at Mikocheni Agricultural Research Institute (MARI). The trial was established using CBSV-free tissue culture material plants of the CBSD susceptible cassava variety Albert, which was micro-propagated using a protocol described by Yona *et al.* (2010). The resulting plantlets were planted on station at Chambezi. Total experimental trial area was 720m$^2$ and established using completely randomized block design (CRBD) with four replications. The trial was established using a completely randomised block design (CRBD) with three replications. Three treatments were used in the study including: inoculums in the center (IC), inoculum diagonally (ID) and inoculums on outer rows (IO) to serve as disease spread sources. A no disease plot served as a control.

Disease assessment was done based on foliar and stem symptoms which were recorded monthly after planting (MAP) with their respective disease incidence and symptom severity. Suitable symptomatic leaves samples were collected as per Rwegasira *et al.* (2011a) and analysed to confirm presence of CBSV using appropriate primers in RT-PCR as described by Monger *et al.* (2001). Also, adult whitefly were counted from the abaxial part of the top five fully expanded apical leaves of a representative shoot on each plant. Counting was done during relative cooler day when insects were less active.

Data collected on spatial and temporal spread of CBSD as well as incidence and severity of the disease, adult whitefly population count were transformed based on statistical requirements. The transformed data were subjected to analysis of variance (ANOVA) and subsequently mean separations were obtained using the Statistical Package for Social Sciences (SPSS 12.0) (SPSS, 2003). The P value was calculated based on foliar and stem severity and whitefly count to determine the significantly within and between replications ($P < 0.005$).

### 3.2 First report of Cassava Brown Streak Viruses Infecting Naturally Growing Non-Cassava Plant Species

Determination of alternative host plants for CBSV in Costal, Tanzania it was done by collection of tender leaves with CBSD-like symptoms from annual weed and shrubs species along all passable roads in the Coast, Dar es Salaam and Tanga Regions. The leaf samples were pressed using herbarium sheets and boards, and left to dry which allowed for longer time of storage prior to detection of CBSVs which was done at Mikocheni Agricultural Research Institute (MARI).

Total RNA was extracted from weed and shrubs leaf samples using CTAB method and complementary DNA (cDNA) generated using RT-PCR method as described Mongel *et al.* (2001). Detection of CBSVs was done using two sets of primers including: CBSDDF and CBSDDR Mbanzibwa *et al*. (2011) and the universal CBSV10 and CBSV11 (Monger *et al*., 2009).

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# PAPER ONE

## Spatial and temporal spread of cassava brown streak disease in field grown cassava in coastal Tanzania

**PAPER TWO**

Expected Journal to be submitted is International Research Journal of Plant science

**First report of Cassava brown streak viruses infecting naturally growing non-cassava plant species**

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**Abstract**

Cassava brown streak disease has been reported to occur in previously un-infected plant stocks. This created a question as to whether there could be plants that acts as alternative hosts to the viruses. The current study aimed at identifying the alternative hosts of *Cassava brown streak virus (CBSV)* and *Ugandan cassava brown streak virus* (UCBSV) collectively known as cassava brown streak viruses (CBSVs). Leaf samples were collected from a total of 97 shrubs and herbs with-virus like symptoms growing in, around or away from cassava fields at 97 locations in the Coast, Dar es Salaam and Tanga Regions. Detection of CBSVs was done at Mikocheni Agricultural Research Institute. Total RNA was extracted from the collected leaf samples and amplified in reverse transcriptase polymerase chain reaction (RT-PCR). Samples from *Annona sophylla Boj* tested positive to CBSV whereas UCBSV was detected in *Solanum incanun L. Elaeodendron duchananii* tested positive for the two viruses (CBSV and UCBSV) suggesting coinfections. This is the first report for CBSV and UCBSV infections in being detected in plant hosts different from cassava. This new findings sheds light on the origin of this viruses that was first reported from East Africa at Amani in Tanga Region. It further suggests that cleaning of new stock from virus might not offer an effective solution to CBSD management in areas where alternative host plants are rampant.

**Key words:** Cassava brown streak disease, alternative hosts, epidemiology and shrubs
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## 4.0 INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub of the family *Euphorbiaceae* that typically grows from one to three meters (3-10 feet) in height (Thresh *et al.*, 1998). Among the 28 known species in the *Euphorbiaceae* family, cassava is the only edible crop and its tuberous roots are a good source of carbohydrates (Abarshi *et al*., 2010; Legg *et al.,* 1998). The crop is a key staple in several African (Alicai *et al*., 2009), South American and Asian countries and has the highest production potential of calories per hectare per day among tropical crops (De Bruign and Fresco, 1989). In most African countries, cassava is grown for food by small scale farmers on subsistence basis and it constitutes an important source of income in rural and often marginal areas, especially for women (Legg *et al.*, 1998). Despite being improtantthe productivity of cassava is hampered by a series of biotic stresses particularly viral diseases namely cassava brown streak disease (CBSD) (Monger *et al., 2001a*, Alicai *et al.*, 2009, Mbanzibwa *et al.*, 2009a and Winter *et al.,* 2010) and cassava mosaic disease (CMD).

Cassava brown streak disease was first reported to be endemic in coastal East Africa at altitudes less than 1000 m.a.s.l (Nichols, 1950). The disease is caused by two viruses: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Both CBSV and UCBSV are members of the genus Ipomovirus in the family Potyviridae and have single-stranded (+) ssRNA genomes that encode a polyprotein (Mbanzibwa *et al*., 2009). Recent efforts have focused on understanding the molecular variability and transmission of the causative viruses of CBSD. Whiteflies (*Bemisia tabaci*) were reported to transmit the two cassava brown streak viruses (CBSVs), albeit at a very low efficiency (Maruthi *et al.*, 2005). A study conducted in Kenya also reported the spiralling whitefly (*Aleurodicus dispersus*) to transmit CBSV (Mware *et al.,* 2009).

Recent efforts geared to understand CBSD have focused greatly on characterising the molecular variability and transmission of the causative viruses (Mbanzibwa *et al.,* 2011; Rwegasira *et al.,* 2011). Whiteflies (*Bemisia tabaci* Genadius) were reported to transmit CBSV albeit at a very low efficiency (Maruthi *et al.,* 2005). The spiralling whitefly (*Aleurodicus dispersus*) has also been demonstrated to transmit the viruses that cause CBSD (Mware *et al.,* 2009). Occurrence of CBSD on originally uninfected stock of cassava crop has been reported (Hillocks, 2003; Kanju *et al*., 2003). However, an epidemiological knowledge gap does exist on the causes for such infection even in crops established away from other cassava crops. Suspected existence of non cassava plants species as reservoirs of CBSVs had been a possibility with no proof. Hillocks (2003) suspected alternative hosts infected with CBSVs to serve as the source of inoculums for new infections to susceptible cultivars when established in the field even if farmers had planted CBSV-free planting materials.

Limited efforts have been geared towards studying the wild plants harbouring CBSV and UCBSV. Although *Manihot glaziovii* which is a close relative to cassava, was recently shown to be infected with CBSV (Mbanzibwa *et al*., 2011) the report did not trigger a quest by scientist on non-cassava relatives that would act as hosts CBSVs. Partial sequences of CBSV isolates from *M. Glaziovii* showed they could be genetically distinct from isolates infecting cassava plants in East Africa which has an implication on the management of the disease. Availability of sequences of isolates from wild species would shed some light on the evolution of CBSV and UCBSV and thus improve our understanding of the adaptation of these viruses in relation to cassava and their new hosts. This is an important aspect especially in predicting the potential of CBSV and UCBSV isolates to evolve into new strains or even distinct viruses. Therefore, this study aimed at linking the knowledge gap on the alternative hosts for CBSV and UCBSV.

## 4.1 Material and Methods

### 4.1.1 Sample collection

Diagnostic survey was made along all passable roads in the Coast, Dar es Salaam and Tanga regions. Sampling interval was 15 kilometres. The car was driven with regular stops after 15 km and assessment was made on vegetation to about 25 meters on both sides of the road. Wherever none of the plants was observed to exhibit virus-like symptoms, another round of 15 km was made until when suspicion sample was observed and collected. Presence of cassava field was used as indicator for possible association of the viruses harboured by the sampled plants with cassava. Leaf samples with virus-like symptoms were randomly collected from the shrubs and herbs growing within cassava fields or in bushes surrounding cassava fields and along the road. Assessment of virus-like foliar symptoms was based on the scale of 1 (no symptoms) to 5 (very severe symptoms) (Rwegasira *et al.,* 2011). A total of 97 leaf samples with clear virus-like were collected. The leaf samples were pressed using herbarium sheets and boards, and left to dry which allowed for longer time of storage prior to detection of CBSVs (Fig. 4.1).

**Figure 4.1: Collected plant samples with virus-like symptoms pressed on herbarium sheet for cassava brown streak virus detection**

### 4.1.2 Detection of the CBSVs

#### 4.1.2.1 Extraction of nucleic acid

Total RNA was extracted using a modified Cety-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). About 200g of symptomatic leaf sample was placed in 1.5 ml eppendorf tubes with metal beads to facilitate grinding for 45 seconds in an automated grinder machine (SPEX SamplePrep, 2010). A total of 700 µl of the extraction buffer mixed with 0.2% β-mercaptoethanol was added and vortexed to disperse the tissue in the buffer. The extract was mixed with an equal volume (700 µl) of chloroform: isoamyl alcohol (24:1) and the contents were mixed and centrifuged at 12 000 rpm for 10 min at 4 ºC. The supernatant (500 µl) was transferred into the new tube and 300 µl cold isopropanol was added to precipitate the RNA. The samples were then incubated at -20 ºC for 10 min after which the chilled contents were centrifuged at 13,000 rpm for 10 min at 4 ºC and the supernatant discarded. The pellets were re suspended in 700 µl of 75% ethanol, incubated at -20 ºC for 10 min and centrifugated for 5 min at 13,000 rpm. RNA was dissolved in 100 µl of RNAse free water.

#### 4.1.2.2 Synthesis of complementary DNA

Reverse Transcriptase Polymarase Chain Reaction (RT-PCR) was performed using a GeneAmp PCR system 9700 thermocycler (Perkin Elmer, Wellesey, Mass, USA). Synthesis of DNA was two step reactions. 1X reaction mixture of total volume of 12 µl contained 9 µl of sterile double distilled water, 1 µl of 10 µM olido (dT18) and 2 µl of total RNA. The reaction was incubated at 70 ºC for 10 minutes and chilled on ice for 5 minutes. Then the master mix (6 µl of sterile double distilled water, 4 µl of 5× RT buffer, 1 µl of 2.5 mM of dNTPs, 1 µl of RNAse inhibitor and 1 µl of reverse transcriptase) was added. The complementary DNA was synthesized by incubating at 42 ºC for 55 minutes.

Reverse transcriptase was inactivated by incubation at 70 ºC for 10 minutes. The cDNA was stored at 4 ºC until used in PCR.

#### 4.1.2.3 Polymerase chain reaction

Detection of CBSVs was done using two sets of primers including: CBSDDF and CBSDDR Mbanzibwa *et al*., 2011) and the universal CBSV10 and CBSV11 (Monger *et al*., 2009). The master mix for PCR contained 12.9 $\mu l$ of sterile double distilled water, 3.0 $\mu l$ of 10X PCR buffer with $MgCl_2$, 2.8 $\mu l$ of 2.5 mM dNTPs, 1 $\mu l$ of each primer, and 0.3 $\mu l$ of 0.5 U Amplitaq Gold Polymarase (Applied Biosystems). Amplifications were undertaken in a gene-Amp PCR system 9700 thermocycler (Perkin Elmer, Wellesey, Mass, USA) under the following conditions: Initial denaturation at 94 ºC for 3 minutes followed by 35cycles at 94 ºC for 30 seconds; annealing at 51 ºC for 30 seconds; initial extension at 72 ºC for 30 seconds and final extension at 72 ºC for 5 minutes. The PCR products were separated by electrophoresis in a 2% agarose gel in 1X Tris Acetate EDTA (TAE) buffer for 1 hour at 80 volts. 1 kb plus DNA marker was used (indicate manufacturer info). Ethidium Bromide was used to stain the amplicons that were visualized under ultraviolet light and images captured using a gel documentation system (BioDoc-IT Imaging System-UVP) (Fig. 4.2).

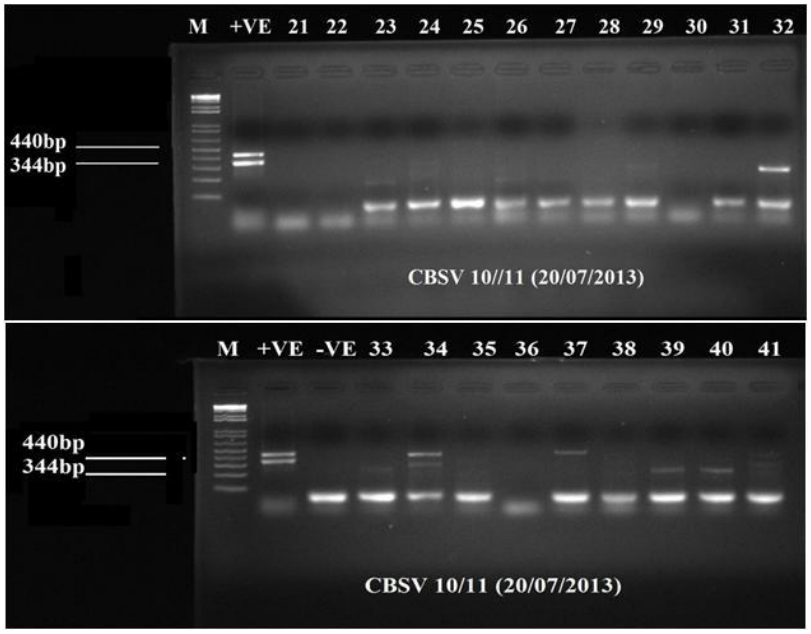


**Figure 4.2: Agarose gel electrophoresis of PCR products from plants with virus-like symptoms**

## 4.2 Results

### 4.2.1 Plant samples and symptoms recorded

The collected plant species (Table 4.1) included all non-cassava plants that were observed to exhibit virus-like symptoms. There was variation in the virus-like symptoms recorded on collected samples. Foliar symptoms included feathery chlorosis on either side of the small veins, yellowing on the older leaves on the apex with intermitted mosaic (yellow and green patches), leaf mottling, leaf curling and spotted yellow patches commonly near the apex. Most of these symptoms appeared on the mature leaves and the young expanding leaves were often symptomless (Fig 4.3).

**Table 4.1: Plant species, geographical distribution and ecology of samples used in the study**

| Plant species | CBSD severity score | Description of ecology samples were collected | Frequency of occurrence in the collection site* | CBSV status |
|---|---|---|---|---|
| *Glycine wightii* Wigh and Arn (Papilionaceae) | 4 | In roadside bushes | Very high | Negative |
| *Albizia versicolor* Welw (Mimosaceae) | 3 | Within cassava fields and in surrounding bushes | Very high | Negative |
| *Vernonia amygdalina* Dalile (Compositae) | 4 | In roadside bushes | High | Negative |
| *Solanum incanum* L. (Solanaceae) | 3 | Within cassava field | Very high | Positive |
| *Psorospermum febrifugum* Spach.var (Clusiaceae) | 3 | In roadside bushes | High | Positive |
| *Dichapetalum stulmannii* Engl (Dichapetalaceae) | 4 | Within cassava fields and in surrounding bushes | High | Negative |
| *Deinbolia borbonica* scheffler (Sapindaceae) | 2 | In bush surrounding cassava field | Low | Negative |
| *Milletia impressa* Harms (Leguminosae) | 3 | In bush surrounding cassava field | Very high | Negative |
| *Tetracera litoralis* Gilg (Dilleniaeceae) | 2 | In bush surrounding cassava field | Low | Negative |
| *Clerodendrum rotundifolium* Oliv(Verbenaceae) | 3 | Within cassava field | Low | Negative |
| *Synedrella nodiflora* Gaertn (Compositae) | 4 | In roadside bushes | Low | Negative |
| *Vigna unguiculata* (L.) Walp (Papilionaceae) | 4 | Within cassava field | High | Positive |
| *Achyranthes aspera L.* (Amaranthaceae) | 3 | Within cassava field | Low | Negative |
| *Commiphora Africana* (A. Rich) Engl.(Burseraceae) | 3 | In roadside bushes | High | Negative |
| *Vidna vexillata* (L.) A.Rich (Papilionaceae) | 4 | In roadside bushes | High | Negative |
| *Disospyros loureiriana* G. Don (Ebenaceae) | 3 | In roadside bushes | High | Negative |
| *Pavetta crassipes* K. Schum (Rubiaceae) | 3 | In bush surrounding cassava field | Low | Negative |
| *Tilianacora funifera* (Miers) Oliv. (Menispermaceae) | 4 | Within cassava fields and in surrounding bushes | Very high | Negative |
| *Annona senegalensis* Pers. (Annonaceae) | 4 | Within cassava fields and in surrounding bushes | High | Positive |

*Frequency of occurrence is categories as follows: Low (<50 plants), High (>50<100 plants) and Very high (>100 plants)


**Figure 4.3: Shrub plants CBSV positive collected with virus-like symptoms A: *Annona senegalensis* Pers. (Annonaceae) B: *Solanum incanum* L. (Solanaceae) C: *Psorospermum febrifugum* Spach.var (Clusiaceae)**

### 4.4.2 Ecology of sample sources

The collected non cassava host plant species were found within the cassava fields, in the surrounding bushes and on the roadside bushes. Some plant species occurred more frequently than others while some were a rare find. The frequency of occurrence were categorized as very high (more than 70% incidence), moderately high (20-50% incidence) and low (less than or equal to 10% incidence) (Table 4.1).

### 4.4.3 Geographical distribution of CBSV and UCBSV infected samples

Detection of CBSVs in the amplified PCR products from plants with virus like symptoms (Fig. 4) indicated the presence of CBSV and UCBSV occurring as single (either CBSV or UCBSV) co-infections (CBSV and UCBSV). The CBSVs were detected in *Annona*

*senegalensis* Pers (CBSV), *Solanum incanun L.* (UCBSV) and *Psorospermum febrifugum* (co-infected). These detections were based on primers CBSDDF/CBSDDR (Mbanzibwa *et al*., 2011) amplification for CBSV (344bp) and UCBSV (440bp) (Table 1.1). The CBSVs positive samples were fairly distributed in the Coast and Tanga Regions (Fig. 4.4).

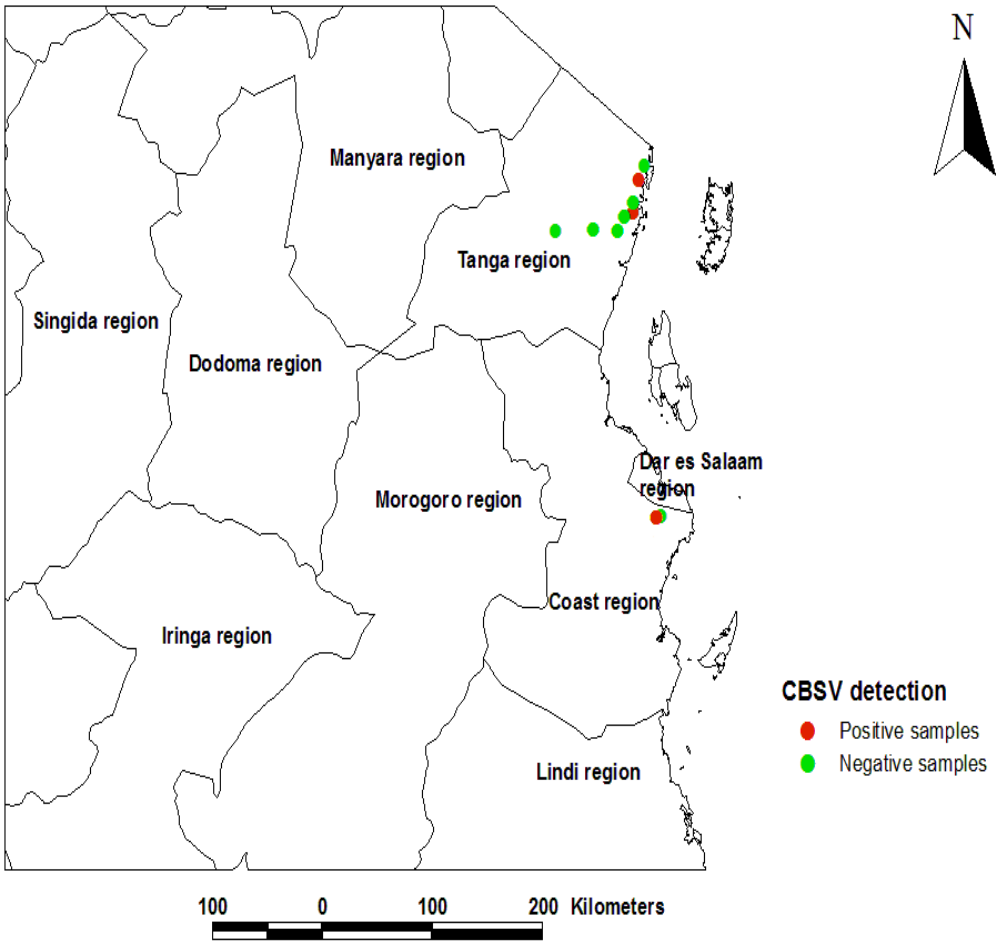


**Figure 4.4: Map showing plants with virus-like symptoms in Coastal, Tanzania, red circles indicate sites from which CBSV-positive samples were collected and green circles indicate sites whose samples were CBSV-negative based on reverse-transcriptase polymerase chain reaction detection.**

**4.3 Discussion**

Results obtained in this study suggest that CBSVs are hosted by several other plant species different from cassava and may be not of the family *Euphorbiaceae*. Although there exist previous studies that indicated other including: *Nicotiana benthamiana* and *Nicotiana debneyi* (Bock, 1994) and Petunia hybrid (*Yolanda Vanveen*) (Mware *et al*., 2010) to host the viruses, the report never struck concerns among epidemiologist because the named plant species are known as virus indicators and reared grow in the wild. Moreover, recent detection of CBSV in *Manihot glaziovii* (Mbanzibwa *et al.,* 2011) did not raise an alarm because the specie is a close relative of cassava. However, the current observation that *Annona senegalensis* Pers*, Solanum incanun L* and *Psorospermum febrifugum* Spach.var are also suitable hosts to CBSVs must raise concerns among epidemiologist and the disease management experts. The probable cause to such diversity of alternative hosts to CBSVs is the large number of plant species that are fed on by whiteflies the proven vector to the viruses and indicated by Mware *et al.* (2010). This new findings possibly answers the question as to why CBSD has very often been found to occur in previously uninfected stock however clean the starting materials would be.

Symptoms from collected shrubs and herbs species from which CBSVs positive samples were collected looked similar to those characteristic of CBSD. These included: yellow chlorosis on the veins chlorotic mottle on the foliar parts, brown necrotic streaks/lesions visible on the green portions of the stems, dark-brown necrotic tissues impregnated in the bases of leaf petioles and irregular senescence. The unique symptoms observed on the sampled shrubs and herbs included leaf mottling and leaf curling. The occurrence of foliar chlorotic blotches and veinal chlorosis were similar to the original descriptions (Nichols1950; Storey's, 1936). These foliar symptoms were observed in all sampled plants from the coast regions that were later confirmed to be infected by CBSVs. The

affected leaves always retained some green patches amidst chlorosis or necrosis. This observation indicates that the symptoms produced by the viruses whether in cassava or other host plants are relatively similar. This confirms the observation by early researchers that several viruses are named and recognized after the symptoms they cause on host plants (Samwel, 1934).

The non-cassava host plant species were diversely distributed ranging from cultivated areas near or within cassava fields to free ranges including areas where other crops are grown such as sweetpotato, maize and pigeon peas. The geographical distribution varied whereby *A. sophylla* and *S. incanun* were mainly found in areas surrounding cassava fields and the roadside in the bushes while *E. duchananii* was found within the cassava fields. As such *E. Duchananii* could be the main alternative host to CBSVs in the cassava-based farming systems while *A. sophylla* and *S. incanun* constitute the incoulum sources of CBSVs in areas never used previously to grow cassava. Thus, *A. sophylla* and *S. incanun* are among the main causes for natural occurrence of CBSVs the phenomenon observed by Kanju *et al*. (2003).

The confirmation of the plants with virus-like symptoms that their hosts of CBSV, RNA was extracted using using 2% of Cety-trimethyl ammonium bromide (CTAB) method as described by Doyle JJ and Doyle JL, 1987 followed by PCR analysis using virus specific primers reported previously (Mbanzibwa *et al.,* 2011). Since this study has based on the PCR detection, further work will be taken to confirm the identity of CBSV by nucleotide sequence analysis to determine relationships with corresponding sequences from cassava.

## 4.4 Conclusion

The current study has closed one of the key gaps in knowledge on the epidemiology of CBSD in Tanzania, and indeed at a regional scale, on the potential role of non-cassava plant species as alternative hosts that acts as reservoir for CBSV leading to subsequent occurrence in originally virus-free stocks. The ability of non-cassava plants to host CBSV suggest the evolutionary closeness confirming Nichols' (1950) suspicion that CBSD sources could be among the naturally growing plants in coastal areas of Tanzania.

## 4.5 Recommendation

CBSD management strategies must not only consider cassava as the host plant but also other alternative plant species that constitutes reservoir for the CBSVs. Further studies should target to explore many other potential alternative hosts and base upon their genetic evolution to predict emergence and spread of new virus specie/strains that may become a threat to cassava and pose great risks to food security.

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# CHAPTER FIVE

## 5.0 CONCLUSIONS AND RECOMMENDATIONS

### 5.1 Conclusions

Cassava brown streak disease was first reported to be endemic in coastal East Africa at altitudes less than 1000 m.a.s.l (Nichols, 1950). The disease is caused by two viruses: *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Both CBSV and UCBSV are members of the genus Ipomovirus in the family Potyviridae and have single-stranded (+) ssRNA genomes that encode a polyprotein (Mbanzibwa *et al*., 2009).

Despite tremendous efforts that have been made on understanding CBSD focused on characterising the molecular variability and transmission of the causative viruses. Natural spread of CBSD in originally uninfected stocks of cassava has been reported (Kanju *et al.*, 2003). However, other the possible existence of other mechanisms of CBSVs spread through farmers and breeders' exchange of planting materials, both locally and internationally remained a challenge. This study is important since it has generated knowledge on epidemiology of CBSD in temporal and spatial spread of CBSD and understanding the role of non cassava hosts plants. A clear understanding of the epidemiology of CBSD is a key help to develop sustainable strategies for the management of the disease in Tanzania and in the wider Africa regions.

## 5.2 Recommendations

Based on the established aspects of CBSD epidemiology, the following are recommendations

i. Use of disease free cuttings for planting materials
ii. Use of resistance varieties as a means of controlling CBSD because the diagnosis is not always straightforward and apparently symptomless plants could be lately affected.
iii. It is no longer possible to eradicate CBSD although it is important tom take common action by all stakeholders to restrict the movement of cassava cuttings through the open quarantine system.
iv. Development of effective and durable management strategies for CBSD by providing extension guidelines to smallholder farmers on management of non-cassava host plants of CBSVs.