MOLECULAR CHARACTERIZATION OF CASSAVA BROWN STREAK VIRUSES IN MOZAMBIQUE

By

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DECLARATION

I certify that this dissertation is my own work and has not been submitted for any degree in any other University. I also declare that this work is the result of my own investigation except, where otherwise identified by references and that I have not plagiarised the works of others.

Signed: 

Jamisse J. G. Amisse

Date: 3 September 2013

DEDICATION

To my dear wife, Carlota Amelia Francisco Tembe, my daughters Shamissa Amisse and Eleny Amisse, together with my dad José Gonçalves Amisse and my mother Maria Manuel Rodrigues Sopas.

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ABBREVIATIONS

ACMV - African cassava mosaic virus

BLAST-Basical local alignment search tool

c- complementary sense

CBSD - Cassava brown streak disease

UCBSV- Ugandan cassava brown streak virus

CBSV - Cassava brown streak virus

CBSaVs - Cassava brown streak associated viruses

CI- Cylindrical Inclusion

CMD - Cassava mosaic disease

CMG's - Cassava mosaic geminiviruses

CMV - Cassava mosaic virus

CP - Coat protein

DAS (ELISA) - Double antibody sandwich ELISA

DNA - Deoxyribonucleic acid

dsDNA - Double stranded DNA

EDTA - ethylenediamine tetra-acetic acid

ELISA - enzyme-linked immuno-absorbent assay

FAO - Food and Agriculture Organization

HC - Pro – Helper Component- proteinase

ICMV - Indian cassava mosaic virus

ICTV - International Committee on Taxonomy of Viruses

IIAM- Instituto de investigaçao Agrária de Moçambique

IITA- International institute of tropical agriculture

INIA- Instituto Nacional de Investigaçao Agrária

MW – Malawi

NCBI- National central for biotechnology information

NIa- Nuclear inclusion a

NIa-Pro - Nuclear inclusion a proteinase

NIb- Nuclear inclusion b

NRI- Natural resources institute

nt - nucleotide

ORF - Open reading frame

P1- first protein

P3- third protein

PAN - Posto Agronómico de Nampula

PCR - Polymerase chain reaction

RAPD - Random amplified polymorphic DNA

rDNA - Ribosomal DNA

RFLP - Restriction fragment length polymorphism

Rpm- revolution per minutes

RNA - Ribonucleic acid

RT-PCR – Reverse transcription- Polymerase chain reaction

SA - South Africa

ssDNA - Single stranded DNA

Tris-HCL – Tris- HCl hydrochloride

µl - microliter

Research outputs

1. Manuscript in preparation

J. Amisse, J. Ndunguru, F. Tairo, N. Cossa, P. Sseruwagi and M.E.C. Rey (2013) Incidence, symptom severity and geographical distribution of cassava brown streak disease and associated viruses in Mozambique (Annals of applied Biology).

2. Conference Proceedings

J. Amisse, J.Ndunguru, F.Tairo, N. Cossa, P. Sseruwagi and M.E.C. Rey (2013) Current status of Cassava brown streak disease and associated viruses in Mozambique. The 12th International Symposium on Plant Virus Epidemiology, 28th January-1 February. Arusha, Tanzania.

CONTENT

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ABSTRACT

Cassava brown streak disease (CBSD) caused by two distinct ssRNA virus species (CBSV and UCBSV of genus *Ipomovirus*, family *Potyviridae*) and transmitted by whitefly (*Bemisia tabaci*), is a major constraint to cassava production in Africa, including Mozambique. In this research, two studies were conducted. First, in order to monitor the incidence, severity and geographical distribution of cassava brown streak disease and associated viruses in Mozambique, field surveys were performed in six cassava major growing provinces. A total of one hundred and fifteen fields and one hundred and forty six fields were surveyed in 2010 and 2012, respectively. The disease was only found in three of six provinces namely Zambezia, Nampula and Cabo Delgado. The CBSD incidence was highest (61.3% and 82.2% in 2010 and 2012, respectively) in Zambezia and lowest (23.6% and 35.1% in 2010 and 2012, respectively) in Cabo Delgado, with cultivars such as Cadri and Robero showing the highest susceptibility to CBSD, while Likonde and Amwalikampiche had relatively low CBSD incidence, illustrating some tolerance to the disease. The results, when compared to previous surveys conducted in 1999 and 2003, demonstrated that the disease is increasing, and replanting new fields with disease-affected cuttings could be responsible for the spread. The second aim of the study was to investigate the genetic diversity of Cassava brown streak viruses, based on analysis of partial sequences of the coat protein gene, in Mozambique. Collections of CBSD-symptomatic leaves were done between June 2010 and June 2012. Diagnostic RT-PCR, using specific primers to screen for the two species, revealed for the first time the presence of *Uganda cassava brown streak virus* (UCBSV) in Mozambique. UCBSV was found in mixed infections with CBSV, and only confined to a single province of Zambézia, while CBSV species were widely distributed. The phylogenetic analysis revealed two subgroups within CBSV, which were 6.7% divergent in nucleotide sequence. The heterogeneity observed among CBSV isolates in Mozambique suggests that in the future studies more sampling is needed to characterize strains and variants. Addtionally, sequencing of the full CP sequence of CBSaVs isolates is required, which may reveal even more diversity. Infectivity assays of cassava brown streak viruses (CBSV and UCBSV) were established using the host indicator plant *Nicotiana benthamiana*. Plant sap was extracted from infected cassava leaves and inoculated into *N.benthamiana* plants. CBSD-like symptoms were observed, and RT-PCR revealed the presence of CBSV in all samples, except for one which was co-infected with UCBSV and CBSV. This study provided further evidence that CBSaVs are efficiently transmitted to *N.benthamiana*. There is scanty information on alternative hosts, therefore more research is needed to identify other potential hosts of CBSaVs in order to develop an effective strategy to control CBSD.

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RATIONALE AND OBJECTIVES

Cassava (*Manihot esulenta Crantz*) is a staple food for more than 700 million people in Africa (FAO, 2006). In Mozambique cassava is the second most important crop after maize (MINAG, 2005). It is a source of carbohydrates as its tubers are rich in starch and the leaves are source of proteins, mineral and vitamins (FAO, 2000; Cock, 1985, Nweke, 1994; Fregene *et al*., 2000; IITA, 2001). Cassava is produced throughout the country, with more emphasis in the Northern provinces (Nampula and Cabo Delgado) and central zone (Zambezia) (Zacarias *et al*., 2003). Cassava is basically grown for human consumption, such as fresh root, soft fresh boiled, dried and processed forms, flour which makes karakata/ugali (personal observation).

Despite its importance, cassava production is limited by several diseases among, which two viral diseases namely: Cassava mosaic disease (CMD) and Cassava brown streak disease (CBSD) are the most important in Mozambique (Zacarias *et al*., 2003). CBSD was reported to be endemic in cassava in Mozambique, particularly in coastal regions of Nampula, Zambezia and Cabo Delgado provinces (Thresh *et al*., 2002). Currently, the disease constitutes a casual factor for the majority of loss in cassava yield, which is estimated to be more than 80% in the farmers fields (Zacarias *et al*., 2004) which consequently contributes to poverty of farmers.

Surveys conducted in 1999, showed that the most affected province was Zambezia with an incidence of 41.3% to 43% (Thresh, *et al.*, 2002; Zacarias *et al*., 2004). Ten (10) years have passed since the last survey was conducted to assess the status of cassava infecting viruses in

Mozambique. It is therefore important to know the disease situation and the causal viruses for CBSD and their distribution in the country.

Two species of CBSaVs exist, namely *Cassava brown streak virus* (CBSV) and C*assava brown streak Uganda virus* (Mbanzibwa *et al*., 2009a; Winter *et al*., 2010). Of the two, CBSV was reported in all countries including Mozambique where the disease occurs, while UCBSV has a more restricted distribution (Mbanzibwa *et al*., 2009a; Rwegasira, 2009). No information is available if the other species (UCBSV) is present in Mozambique, and therefore, it is critical to establish the CBSD causing virus species and their genetic diversity in Mozambique. This information is important for predicting the disease impact and devising appropriate and reliable control strategies for management of CBSD.

Objective of study

The overall objective of the study was to establish the occurrence, genetic diversity and distribution of CBSD and associated viruses in Mozambique.

Specific aims:

1. To establish incidence, symptom severity and geographical distribution of cassava brown streak disease (CBSD) and associated viruses in Mozambique.

2. To determine the genetic diversity of *Cassava brown streak virus* and *Ugandan cassava brown streak virus* in Mozambique.

CHAPTER ONE

LITERATURE REVIEW

1.1. Cassava the Crop

1.1.1. Origin of cassava

Cassava (*Manihot esculenta* Crantz) is a perennial root crop, with a plant height ranging from 2 to 4 meters. It is believed to have originated from Central and South America (Scott *et. al.,* 2000) and introduced into the African continent in the 16th century by European traders (Scott *et al.*, 2000). In Mozambique, cassava was introduced by the Portuguese in the 17th century (Woodward *et al*, 1997). Fauquet and Fargette (1990) reported that cassava is cultivated in more than 80 countries between 30^{o} south and 30^{o} north of the equator, which indicates its adaptability to a wide range of ecosystems.

1.1.2. Importance of cassava

Cassava is consumed by an estimated 600 million people (FAO, 2002). In Africa, cassava is primarily produced for human consumption, either fresh, dried or pounded, and is also consumed in various processed forms such tapioca, rale, biscuits, sausage rolls, gari, fufu (cassava bread), meat pies, bread, ethanol for energy, cooking and in beer production industries (Cleanstar, 2012; SAB Miller, 2012). In addition to human consumption, some cassava is processed for animal feed and industrial products, either fresh or in various processed forms (FAO, 2006). Starch extracted from cassava root is used in industries as a

raw material for a wide range of food products and industrial goods, including paper, cardboard, textile, plywood, glue and alcohol (FAO, 2000). Another important source of income for farmers is that stem cuttings of cultivars that are used for commercialization (Alves, 2002) can be sold, where each stem may fetch around USD 0.15 in Mozambique (personal observation).

Cassava contains more calories than potato and is a highly productive crop in terms of food calories produced per unit land area per unit of time, significantly higher than other staple crops. Cassava can produce food calories at rates exceeding 250,000 cal/hectare/day compared with 176,000 for rice, 110,000 for wheat, and 200,000 for maize (corn) (http://en.wikipedia.org/wiki/cassava).

Additionally, the roots are rich in calcium and vitamin C and contain a nutritionally significant quantity of thiamine, riboflavin and nicotinic acid. Cassava starch contains 70 percent amylopectin and 20 percent amylose. Cooked cassava starch has a digestibility of over 75 percent (http://en.wikipedia.org/wiki/cassava). However, cassava has antinutrional and toxic factors, and some cassava cultivars have roots that contain large amounts of cyanohydrins (cyanogenic glucosides) that originate hydrogen cyanide, a toxic to human health. Usually most of the bitter taste varieties are rich incyanohydrins agents (DU *et al*., 1995; Andersen *et al.*, 2000).

1.1.3. Cassava production

In 2002, world cassava root production was estimated to be 184 million tonnes rising to 230 million tonnes in 2008, and Africa was a major contributor followed by Asia and Latin

American (FAO, 2009). More than 40 countries in Africa produced cassava, and the top 10 countries leading the production in Africa, namely Nigeria, Angola, Madagascar, Uganda, Benin, Democratic Republic of Congo, Ghana, Malawi, Mozambique and Tanzania (FAOSTAT, 2008).

Mozambique is the 5th highest cassava producer in Africa and the 10th in the world, with 6149897 ton per year (FAO, 2000; FAO, 2004). Approximately 2.5 million farms in Mozambique cultivate cassava, and almost 12 million people use cassava for consumption (MIC and FAO, 2007). The main cassava producing provinces in the country are Nampula (46.7%), Zambézia (25.2%), Cabo Delgado (13.4%) and Inhambane (7.4%) and all these provinces contribute to approximately 93% of the national production (FAO, 2000; MIC and FAO 2007). The nation-wide yield average per hectare recorded in the farmers field is around 8-10 ton, lower compared with world yield average (12.5 ton/ha) and lowest in comparison with the most productive cassava farms-India (34.8 ton/ha) (http://faostat.fao.org in "FAOSTAT: Production, Crops, Cassava, 2010 data". Food and Agriculture Organization. 2011).

1.1.4. Cassava propagation

Cassava is propagated by seed and cuttings (IITA, 1990). The propagation by cuttings is used by farmers and the seed propagation is most commonly used in breeding programs. The use of stem cuttings constitutes a risk to the crop in the proliferation of virus disease problems, unless the source of planting material is carefully selected (Thresh *et al*., 1994).

1.1.5. Production constraints

Cassava is infected by many pathogens such as fungi, bacteria, viruses and mycoplasma (Lozano and Booth, 1974). Some pests such as cassava mealybug and cassava green mite are also considered as major constraints to cassava production (Hahn *et al*., 1980; Nweke *et al*., 2002). Worldwide, at least 17 different viruses have been reported that infect cassava (Thresh *et al*., 1994; Fauquet *et al*., 2005). Viruses are the main biotic constraint to cassava production in Africa, and 8 viruses of 17 reported are known to infect cassava in Africa, *Cassava brown streak virus* (CBSV), *Ugandan cassava brown streak virus* (UCBSV) and Cassava mosaic begomoviruses (CMBs) are the major species leading to constraint of cassava production in Africa, including Mozambique (Calvert and Thresh *et al*., 2002).

1.2. Cassava Brown Streak Disease (CBSD)

1.2.1. History and distribution of CBSD in Mozambique

Cassava brown streak disease (CBSD) was reported for the first time in Usambura Mountain, Tanganyika (now Tanzania) (Storey, 1936). Nichols (1950) reported that disease was endemic in all coastal areas of East Africa (Tanzania and Kenya) and at low altitudes of Malawi (Hillocks and Jennings, 2003). Most of surveys reported that CBSD was found at lower altitudes and was absent at high altitudes above 1000 m above of sea level (Storey, 1939; Nichols, 1950). In the recent outbreaks of CBSD it was observed that the disease may also occur at higher altitudes around Lake Victoria in Uganda and Tanzania (Alicai *et al*., 2007; Rwegasira, 2009) and Burundi (Bigirimani *et al*., 2011).

In 1997, farmers in the major cassava growing areas in northern Mozambique (Nampula, Cabo Delgado and central Zambezia provinces) reported major losses of cassava due to root necrosis by CBSD. In 1998 the losses were considerable, with large areas of coastal Nampula province infected by the disease (Thresh *et al*., 2002). A survey conducted in 1999 revealed that the disease was only present in Nampula, Zambezia and Cabo Delgado provinces, with incidence raging from 23-43 % (Thresh *et al*., 2002).

1.2.2. Aetiology of Cassava Brown Streak Disease (CBSD)

For a considerable time some uncertainty existed with regard to the aetiology of CBSD, and it was assumed to be caused by a virus when the disease was successfully graft transmitted (Storey, 1936; Nichols, 1950). The first real evidence of the causal agent for CBSD was the confirmation of mechanical transmission of the virus in a range of herbaceous indicator hosts done by Lister (1959) and in later experiments by Bock and Guthrie (1976). Later on, virus particles were detected by electron microscopy in leaf samples showing typical CBSD symptoms (Hillocks, 2000). Initially, it was thought that CBSD was caused by a complex of two viruses (Lennon *et al*., 1986). Harrisson *et al*. (1995) observed pinwheel inclusions typically produced by viruses of the *Potyviridae* family, however the separation of single viruses failed. Molecular studies conducted by Monger *et al*. (2001a) showed that CBSD was caused by CBSV alone. However, more recently, the characterization based on a comparison of coat protein (CP) and full length sequences demonstrated that CBSD is caused by isolates of at least two phylogentically distinct species of single stranded RNA (ssRNA) filamentous virus particles (Fig. 1 A) belonging to the *Potyviridae* family and genus *Ipomovirus* (Mbanzibwa *et al*., 2009b; Mbanzibwa *et al*., 2011 and Winter *et al*., 2010).

Cassava brown streak virus (CBSV) is predominantly found in the coastal lowlands of Mozambique and Tanzania, while *Uganda cassava brown streak virus* (UCBSV) occurs mainly in the highland regions of Uganda and Tanzania (Mbanzibwa *et al*., 2009a; Winter *et al*., 2010).

1.2.3. Description of family *Potyviridae*

The name *Potyviridae* comes from "POTATO VIRUS Y GROUP" (Gotz *et al.*, 1995 and Jansen *et al.*, 2005). CBSV and UCBSV are members of the genus *Ipomovirus* and family *Potyviridae* (Mbanzibwa *et al*., 2009b; Winter *et al*., 2010). The family *Potyviridae* comprises about 200 members, 20% of which are plant viruses (Chen *et al.*, 2001). The *Potyviridae* family is one of the largest and economically most important families of plant viruses due to their effects on crops worldwide.

Based on their transmission, the family *Potyviridae* consists of 8 (eight) genera about_40% of the known plant RNA viruses (Adams *et al.*, 2011). *Bymovirus* transmitted by fungi, *Ipomovirus* by whiteflies, *Macluravirus* and *Potyvirus* by aphids, *Rymovirus* and *Tritimovirus* by mites (Chen *et al.*, 2001), *Brambyvirus*, type member *Blackberry virus Y* (Susaimuthu *et al*. 2008; Carstens, 2010) by unknown aerial vectors and *Poaceavirus* (type member *Triticum mosaic virus; Sugarcane streak mosaic virus*) (http://www.dpvweb.net/notes/showgenus.php?genus=Poacevirus).

All members of family *Potyviridae* have filamentous particles of 650-900 nm in length (Shukla *et al.*, 1998; Chen *et al.*, 2001), except the bymoviruses, which are bipartite and 500-600 nm length and 11-13 nm wide (Shukla *et al.*, 1994; Agrios, 2005). The coat proteins of the

potyviruses are made up of about 2000 units of a single structural polypeptide and the genomes consist of a linear, single-stranded positive sense monopartite RNA genome of 8500-12000 nt with a poly(A) tail at the 3'- terminus and a genome linked protein (VPg) at the 5'-terminus (Chen *et al.,* 2001).

The characteristic *Potyviridae* structures such as cylindrical inclusion (CI) bodies can be seen in the infected cells of plants (Ward and Shukla, 1998; Gibbs and Mackenzie, 1997). A few members induce cylindrical nuclear inclusions (CNI), which contain a small proteinase (NIa) and large Nuclear inclusion (NIb) polypeptide, while other members induce the formation of non-crystalline amorphous inclusions containing the helper-component proteinase (HC-Pro) proteinase (Shukula *et al*., 1994), except the genome of Cassava brown streak viruses where it was observed that the CBSV genome lacked HC-Pro found in potyviruses (Mbazinbwa *et al*., 2009b).

1.2.4. Genetic variation and genome organization of CBSD causal viruses

The viral genome of CBSV , the first species of ipomoviruses to be identified, is monopartite with a linear, positive sense, single strand RNA genome made of about 9 000 nucleotides, predicted to produce a polyprotein of 2902 amino acids *(*Mbanzibwa *et al*., 2009b; Winter *et al*., 2010). Lecoq *et al*. (2000) demonstrated that the virion structure is a filamentous particle with an elongated helical symmetry (flexuous filamentous rod), 650-690 nm in length. The genome of CBSV encodes P1 serine proteinase (the first protein which is located upstream of P3 protein). It was observed that the CBSV and UCBSV genome lacked the HC-Pro found in all other members of the family *Potyviridae* except CVYV and SqVYV, but that the structure of the 3'- proximal part of the genome was shown to contain a unique Maf/HAM1-like

sequence (678 nucleotides, 226 amino acids) recombined between the replicase and highly conserved coat protein (CP) domains. Ipomoviruses are exceptional in their variability of protein-encoding sequences at the 5'-end of the genome (Fig. 1 B) (Mbanzibwa *et al*., 2009b). Two other ipomoviruses, *Cucumber vein yellowing virus* (CVYV) and *Squash vein yellowing virus (*SqVYV*)*, were observed to lack HC-Pro, but have two P1 proteinases. The P1 of CBSV (362 aa) is most closely related to the P1 of *Sweet potato mild mottle virus* SPMMV and P1b of CVYV and SqVYV, which all are related to the P1 of tritimoviruses (Mbanzibwa *et al*., 2009 b). HAM-1 like protein has been identified in *Euphorbia ringsport virus* (EuRSV, *Potyvirus*) between the NIb and CP regions (ICTVdB, 2005). The properties of CBSV are similar to that *Sweet potato mild mottle virus* (SPMMV) *(*Colinet *et al.,* 1996) and *Cucumber vein yellowing virus (*Lecoq *et al.,* 2000), members of the *Potyviridae* family and possibly the *Ipomovirus* genus (Monger *et al*., 2001a).

Different strains of the CBSD causal viruses have been reported based on partial CP sequences (Mbanzibwa *et al*., 2009a; Rwegasira *et al*., 2011) and recently Winter *et al*. (2010) have shown that there are two distinct phylogenetic groups, based on full length sequences. Mbanzibwa *et al* (2011a) reported that the CP of the two viruses differ in size since the region coding for the CP-N-terminus in UCBSV is lacking 33 nt (11 amino acids) as compared to CBSV. The 3-untranslated regions (3'-UTR) of the two viruses differ in length of 94–96 nucleotides (Mbanzibwa *et al.,* 2009a).

Comparison of the complete genome between CBSV isolates with UCBSV isolates indicated that there was 69.0-70.3% and 73.6-74.4% similarity at the nucleotide and polyprotein amino acid sequences, respectively, and among the CBSV isolates the variation was wider (79.3-95.5% at nt level) compared with UCBSV isolates (86.3-99.3%) (Mbanzibwa *et al*., 2011b).

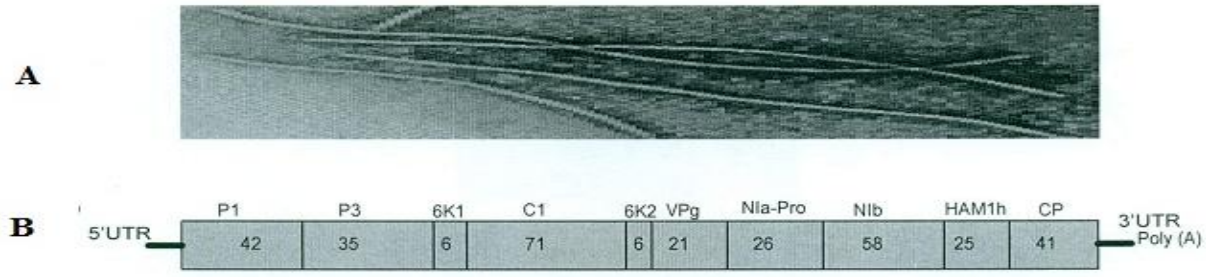

Figure 1 : A= Particle morphology of CBSV (Lenon *et al*., 1986); B=Schematic representation of CBSV genome (Mbanzibwa *et al*., 2009b).

1.2.5. Transmission of cassava brown streak associated viruses (CBSaVs)

In Tanzania, seeds were collected from plants showing leaf symptoms of CBSD and seedlings were established. Cassava plants were grown under controlled screen house conditions, and after six months all the plants did not show any symptoms of CBSD. The absence of CBSV was confirmed in 30 plants randomly selected and tested by RT-PCR (Maruthi *et al*., 2005); supporting the evidence that CBSV is not transmitted by seed propagation. To date, no study has reported the transmission of CBSD causal viruses by seed propagation, so the cassava seeds may be exchanged regionally and internationally without risk of spreading CBSD (Rwegasira, 2009). Vegetative cassava propagation using cuttings is the most important means of CBSD transmission.

Since 1939 the whitefly *Bemisia tabaci* has been suspected as the vector for CBSD (Storey, 1939). Robertson (1987) reported the correlation between numbers of whitefly in the many fields of East Africa and the CBSD symptoms of plants. Another study conducted by Bock (1994) reported that CBSV is insect transmitted and the most probable insect is the whitefly *B. tabaci.* In Tanzania it was observed that in some field, that spread of infections in cassava

plants with CBSD coincided with increases of whitefly numbers, and indeeded that *B. tabaci* is a vector of CBSV (Maruthi *et al.,* 2005).

Experiments using inoculation of mixed population of *B. afer* and *B. tabaci* in cassava plants showed that the transmission of CBSV from *B. tabaci* exhibited low efficiency (20-22%), however typical symptoms and infection of CBSV were observed, and virus infections were confirmed by RT-PCR (Maruthi *et al.,* 2005). Furthermore when 2 whiteflies were separated the transmission was achieved only with *B. tabaci* (Maruthi *et al*., 2005). CBSV has also been shown to be transmitted by spiraling whitefly (*Aleurodicus dispersus).* In experiments with *B.tabaci* and *A. dispersus* the rate transmission of CBSV by *B.tabaci* was 40.7% when compared to that of *A. dispersus* (25.9%) (Mware *et al*., 2009).

1.2.6. Host range of CBSaVs

The disease, CBSD, only occurs in Africa, and has not been reported in America and Asia and it probably that the viruses originated from wild host plants species native to Africa. The natural occurrence of disease is in the cassava tree (Family: *Euphorbiaceae*, Genus *Manihoti*, specie *Manihot esculenta* Crantz) (Storey, 1939). There are reports of a close relative of cassava being alternative host of CBSV in Kenya (Bock, 1994) and *M.glaziovii* was found infected with CBSV isolates in Tanzania (Mbanzibwa *et al.,* 2011a).The following plant species have been used as experimental hosts for CBSV: *N. benthamiana N. tabacum, N. debneyi, N. rustica, N. glutinosa, N. hesperis, N. occidentalis, N. occidentalis ssp., Datura stramonium, Petunia hybrida, Chenopodium quinoa* and *C. amaranticolor* (Thresh, 1994; Bua and Namara, 2008). Of these species, *N.debneyi* and *N. benthamiana* have proved the most useful (Bock, 1994; Monger *et al.,* 2001b; Winter *et al*., 2010). Bua and Namara (2008)

conducted studies in screen house at Kyambogo University using different species of *Nicotiana*, and observed that all species showed symptoms (general chlorosis, vein chlorosis and necrotic lesions), where *N.benthamiana* and *N. tabacum* recorded the highest level of CBSD and *N.glutamia* the lowest level.

1.2.7. Symptomatology of CBSD in cassava

All parts of the cassava plant (leaves, stem and roots) may show symptoms of CBSV infection, but the aspects of the symptoms and its degree, vary greatly and depend on environmental conditions, the growth stage of the crop, relative humidity, the time of infection and the sensitivity of the cultivar (Nichols, 1950; Hillocks and Thresh, 1998; Hillocks *et al.*, 1999).

Two types of foliar symptoms associated with CBSD can be distinguished: type 1 appears firstly as chlorosis along the margins and later tertiary veins; type 2, chlorosis which develops in areas between the main veins and later cover much of the leaf lamina. CBSD leaf symptoms appear on the mature leaves, which is not common in majority of plant viruses' symptoms (Nichols, 1950).

Root symptoms are the most destructive and usually develop after foliar symptoms. They are characterized by root necrosis, and some cultivars may show root symptoms eight months after planting, but this varies between cultivars. In the most susceptible cultivars, root necrosis may appear within six months of planting cuttings derived from symptomatic mother plants (Hillocks *et al*., 2001; Hillocks *et al*., 2002). The time taken between the appearance of foliar symptoms and the development of root necrosis depends on varietal characteristic. Root symptoms are variable on the outside of the root and may appear as radial constrictions and/or

pits and fissures in the surface bark. Tissue surrounding the pits is stained brown or black. Below the pits, the cortex is necrotic. The internal symptoms consist of a yellow/brown, corky necrosis of the starch-bearing tissue, sometimes with blue/black streaks. Sometimes, the roots appear healthy on the outside with no obvious constrictions or size reduction, but when cut open, they are found to be necrotic (Hillocks *et al.*, 2001; Hillocks *et al*., 2002; Legg and Thresh, 2003).

An interesting aspect of this disease is variability of symptoms. For example, leaf and/or stem symptoms can occur without the development of tuber symptoms (Hillocks *et al*., 1997). Furthermore, three isolates from Tanzania were found to exhibit different symptoms on the secondary host plants *N. benthamiana* and *N. tabacum* (Monger *et al*., 2001a).

Figure 2: CBSD symptoms (a) foliar chlorosis; (b) stem necrosis and (c) root necrosis

1.2.8. Strategies to control CBSD in Mozambique

Earlier research conducted by Cassava National Program of Mozambique Research Agriculture Institute (IIAM) to control the disease was based on the identification of cassava landraces tolerant to CBSD. Four varieties (Baadge, Kigoma mafia, Nachinyaya and Likonde) were

identified as tolerant to CBSD. After identification of these varieties, many NGO's, namely: CARE, SAVE THE CHILDREN, International Institute of Tropical Agriculture (IITA) and SARRNET developed activities based on the multiplication and dissemination of the varieties. Further response to the disease situation led to additional activities carried out by the national program, and firstly was to teach farmers phytossanitary measures to reduce the spreading of disease, and secondly was the Breeding program, whose main objective was to develop and improve new cassava varieties tolerant and resistant to both viral disease (CBSD and CMD). Under the breeding program, in 2009, five tolerant varieties to CBSD and CMD were released. Additionally, current studies are continuously carried out looking for enhancement of the strategy to control the viral diseases in Mozambique. Surveys conducted in the 3 provinces, Nampula, Zambezia and Cabo Delgado in May 2010 and 2012, revealed that among all cultivars surveyed, two cultivars, namely Tomo and Robero, never showed symptoms of CMD even in the fields where the disease was severe, which suggests that the cultivars are tolerant or resistant. Furthermore, to explore and understand their tolerance, the two cultivars were sent to NRI, Greenwich University (UK) for tissue culture and in the future will be tested regionally to evaluate their tolerance and resistance to CMD.

1.2.9. New approaches to control viruses

The lack of durable natural resistance to CBSD, in the cassava germoplasm grown by farmers, requires the development of transgenic varieties as one of the strategies to control the CBSD pandemic. One of the most promising transgenic methods to confer resistance in cassava against CBSD is the use of RNA intereference (RNAi) (UNCST, 2009; Vanderschuren *et al*., 2012) and some transgenic lines have been developed using this method, and most of them showed high level of resistance to CBSV/UCBSV under laboratory condition (Vanderschuren *et al*., 2012). However the lines need to be tested under

field conditions to determine the effectiveness of the genes. In Uganda, some transgenic lines against CMD have been developed by scientists from the Cassava Research team at NaCRRI and are underway to be evaluated under natural field condition (UNCST, 2009).

CHAPTER TWO

INCIDENCE, SYMPTOM SEVERITY AND GEOGRAPHICAL DISTRIBUTION OF CASSAVA BROWN STREAK DISEASE AND DETECTION OF ITS CAUSAL VIRUSES IN MOZAMBIQUE

2.1 Abstract

A study was conducted to establish the incidence, symptom severity and geographical distribution of cassava brown streak disease (CBSD) and causal viruses in six major cassava-producing provinces of Mozambique in 2010 and 2012. In addition, laboratory analyses were conducted using species-specific RT-PCR primers to determine the viruses causing CBSD in Mozambique. Typical CBSD symptoms were recorded in only three of the six provinces surveyed, namely Zambezia, Nampula and Cabo Delgado. One hundred and fifteen (115) fields were assessed in 2010 in Cabo Delgado (33), Nampula (41) and Zambezia (41), while one hundred forty six (146) fields were surveyed in 2012 in Cabo Delgado (46), Nampula (44) and Zambezia (56). Sampling was done along two diagonals across each field by scoring 30 plants per field. Field assessments were conducted for CBSD incidence, symptom severity, virus species and geographical distribution. In the 2010 and 2012 surveys, CBSD incidence was highest in Zambezia (61.3% & 82.2%) and lowest in Cabo Delgado (23.6% & 35.1%). More than 50% of the assessed fields in 2010 and 40% in 2012 had a mean CBSD incidence of 76-100%. Disease foliar symptom severity score varied between 2.4 to 2.8 in all three provinces. Most of the cultivars were found to be susceptible for CBSD with incidence ranging from 0 to 98.7%. CBSD incidence was highest on cultivars Cadri (98.7%) and Robero (96%) and lowest on cultivars Likonde (0%) and Amwalikampiche (16.7%). RT-PCR results produced amplicons of the excepted size (344 bp) for CBSV isolates, confirming the occurrence of CBSD in all three surveyed provinces.

2.2. Introduction

Cassava (*Manihot esulenta Crantz*) is a major staple for more than 200 million people in sub-Saharan Africa (Dahniya, 1994) including approx. 21 million people in Mozambique (Zacarias, 2008). However, its production is hampered by two viral diseases: cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) (Thresh *et al.*, 1997, Legg *et al.*, 2006, 2011). CMD was reported to cause yield losses of between 19 and 27 metric tones, estimated at more than USD 1.5 billion annually (Thresh *et al*., 1997; Legg and Thresh, 2004). In contrast, CBSD was reported to cause losses up to 70% yield in the farmers' fields. Recent estimates put the losses due to CBSD above USD 100 million annually (IITA, 2005; Hillocks *et al.*, 2001).

Cassava brown streak disease is caused by two positive sense ssRNA viruses (genus *Ipomovirus*, family *Potyviridae*) named: *Cassava brown streak virus* (CBSV) and *Ugandacassava brown streak virus* (UCBSV) (Mbanzibwa *et al*., 2009a; Winter *et al*., 2010). It has been reported that CBSaVs are insect transmitted by *Bemisia tabaci* (Maruthi *et al*., 2005) and the spiraling whitefly (*Aleurodicus dispersus*) (Mware *et al*., 2009). In all cases, however the rate of transmission was very low. In addition, CBSaVs are transmitted through planting CBSD-affected planting material.

Symptoms of CBSD are characterized by vein clearing and chlorosis on leaves, brown streaks on the stems, and constriction and necrosis in the tuber (Storey, 1936) rendering them unfit for consumption.

Contrary to earlier suggestions that CBSD was a low altitude disease restricted to coastal Kenya, Tanzania, Mozambique and in the low lands of Malawi (Hillocks and Jennings, 2003 and Rwegasira, 2009), the disease has been found to occur at higher altitudes such as in the Democratic Republic of Congo (DRC) (Mahungu *et al*., 2003), Uganda (Alicai *et al*., 2007), the Lake Victoria Zone of Tanzania (Jeremiah and Legg, 2008) and Burundi (Bigirimana *et al.*,2011). This has caused devatsting effects to the cassava crop in these areas, threatening the livelihoods of many cassava dependant rural communities.

In Mozambique, CBSD was reported to cause high yield losses on cassava in the 1990s with an incidence of 43% in Zambezia province in 1999 (Thresh, *et al.*, 2002; Zacarias *et al*, 2004). Since then, not much has been done to assess the disease in the country. Current information is therefore lacking on the extent of spread of the disease and the associated viruses (CBSaVs) in the country. Therefore, the aim of this study whose results are described here, was to establish the occurrence and geographical distribution of CBSD and the associated viruses in Mozambique.

2.3. Materials and methods

2.3.1. Study area and field sampling

Two field surveys were conducted in Nampula, Zambezia and Cabo Delgado, three major cassava producing provinces of Mozambique in 2010 and 2012. Nampula is a province of northern Mozambique, mainly a highland area with rugged mountains and Miombo forest and altitude ranging from 9-570 m above sea level (a.s.l). The climate is tropical with an annual rainfull average of 1000 mm, an average temperature of 26ºC and relative humidity ranging between 50-75%. Cabo Delgado is the north-eastern province of Mozambique, has an average altitude of 26-875 m a.s.l and runs west along the east coast along the Indian Ocean, the north bordering Tanzania separated by Ruvuma River, and average temperature of 25ºC. Zambezia province is located in central Mozambique, with an average altitude of 4-716 m a.s.l, and average rainfull of 1500-2000 mm. The prominent agroecology shared by all three regions/provinces is mainly savannah short grassland although Zambezia has considerable forest cover inland.

Sampling was conducted by selecting cassava fields randomly at 10 to 15 km intervals along main roads traversing each province. Cassava fields, aged 3 to 9 months after planting (MAP) were sampled for CBSD. Geo-coordinates of the sampled areas were recorded using a Geographical Positioning System (GPS) and maps were generated using ArcGIS 9.0 (ESRI, 2006).

2.3.2. Field disease assessment

In each field, 30 cassava plants were assessed along two diagonals (making an 'X') for CBSD incidence and severity of CBSD were recorded based on foliar observable symptoms alone. Only a few plants were uprooted for root symptoms observation. CBSD incidence was assessed as the number of plants with disease symptoms expressed as a percentage of the total assessed (Fauquet and Fargette 1990). CBSD symptom severity was assessed using 1 to 5 scale, where 1 represents symptom-free (no aparents chlorosis symptoms characteristic for CBSD on the leaves), 2 (slight chlorotic spots/blotches seen on the leaves), 3 (CBSD chlorotic spots/blotches easily observable on leaves), 4 (appreciable CBSD chlorotic spots/blotches seen on leaves) and 5 severely diseased plants (very severe chlorotic/necrotic blotches and leaf wilt) (Hahn *et al*., 1980 and Hillocks *et al*.,1996). The disease assessements were done of the most common cassava cultivar in each field where mixed cultivars were recorded.

2.3.3. Collection of virus isolates and RNA extraction

Mature fresh leaves with typical CBSD symptoms were collected at each site and kept in a plastic bag on Nitrogen ice during collection and in the laboratory the samples were kept in the freezer at -80^{o}C for RNA extraction and molecular characterization. RNA was extracted using a modified CTAB protocol (Mbanzibwa *et al*., 2009a).

2.3.4. RT-PCR amplification of CBSaVs

Total RNA obtained from 2.3.3 extracted was used to synthesize cDNA in 2 steps using ImProm-II TM reverse transcriptase Kit (Promega, UK) following the manufaturer's instructions. Synthesis was performed in a total of 25 µl as follows description: First step, MASTER MIX 1 consisted of 9 µl of sterilized distilled (sd) water, 1 µl of oligo dT (10 µM) and 4 µl of RNA template. The mixture was incubated by heating at 70^{o}C for 5 minutes and immediately chilled on ice for 2-5 minutes. Step 2, MASTER MIX 2 consisted of 6.5 µl of sd water, 4 µl of 5X RT buffer, 1.0 µl of dNTPs (2.5 µM), 0.5 µl of RNAse inhibitor and 1.0 µl of reverse transcriptase. The reaction was mixed gently and 13 µl aliquotes were dispensed into each 12 µl reaction tube containing MASTER MIX1 making up a total volume of 25 µl. The reverse transcription was performed in the following cycle: 42^{o}C for 60 min, 70^{o}C for 10 minutes, and the resulting cDNA synthesized was used for PCR. The remaining cDNA was stored at -20^{o}C for further analysis.

PCR was run for cDNAs obtained from cassava samples using the CBSDDF2 (5-GCTMGAAATGCYGGRTAYACAA-3) and CBSDDR (5-GGATATGGAGAAAGRKCTCC-3) primers to amplify partial 344 bp fragments of the coat protein (CP) of CBSV and a 430-440 bp fragment for UCBSV (Table 1). The PCR reaction mix consisted of 25 µl made up of 12.9 µl sterile de-ionized water, 3.0 µl of 10 X PCR buffer + 20 mM $MgCl_2$, 1.0 µl of each of the primers CBSDDF/CBSDDR (10 mM), 0.3 µl of U *Taq* DNA polymeramase, 2.8 µl of 2.5 mM of deoxynucleotide triphosphate (dNTPs) and 4.0 µl of cDNA template.

PCR cycling programme was performed using the thermocycler ICycler Biorad® version 4.006 as follows: 94 ^{o}C for 2 min for initial denaturation followed by 35 cycles of 94 ^{o}C (30 sec), 51 ^{o}C (30 sec) and 72 ^{o}C (30 sec) for denaturation, annealing and extension, respectively. PCR products were analyzed by electrophoresis in a 1×TAE buffer on a 2% agarose gel, stained with ethidium bromide (0.1 mg/ml).

Table 1: Primers for PCR used to detect Cassava brown streak viruses (CBSV and UCBSV) in CBSD-symptomatic leaves collected during the 2010 and 2012 country wide surveys in Mozambique

Primer*	Sequences (5'-3')	Specificity	Strand	Target	Prod. size (bp)
CBSDDF2	GCTMGAAATGCYGGRTAYACAA	CBSV and UCBSV	Sense	CP	344 for CBSV and 440 for UCBSV
CBSDDR	GGATATGGAGAAAGRKCTCC	CBSV and UCBSV	Antisense	CP	344 for CBSV and 440 for UCBSV

*Cassava brown streak viruses (CBSaVs) specific primers used for the study as described by Mbanzibwa *et al.,* (2011a)

2.3.5. Statistical analyses of data

Data on CBSD incidence, symptom severity were summarized as percentage incidence and mean severity. Scores for symptom-free plants were omitted when calculating the mean severity. The data were subjected to a one-way analysis of variance (ANOVA-1) using the GenStat 14.0 statistical package.

2.4. Results

2.4.1. Cassava brown streak disease (CBSD) symptoms

During the two field surveys a range of CBSD symptoms were observed on the affected cassava plants (Fig. 3). Foliar (leaf) symptoms comprised of chlorosis and vein clearing, stem

symptoms were necrosis, while root symptoms (on the few plants that were uprooted) comprised of necrosis on the edible flesh (Fig. 3D & E).

Figure 3: Field symptoms of CBSD affected cassava leaves with chlorosis (A & B), stems with necrosis (C) and root necrosis (D & E)

2.4.2. CBSD incidence

There were significant differences in the incidence of CBSD between provinces in both 2012 and 2012 (Table 2). Incidence of CBSD was highest in Zambezia (61.3% & 82.2%) and lowest in Cabo Delgado (23.6% & 35.4%) in both 2010 and 2012 (Table 2 and Fig. 4). Interestingly, the incidence of CBSD was similar in Nampula province in 2010 (48.3%) and 2012 (48.3%). On average 44.3% & 34.9% and 47.4% & 65.0% of the cassava fields surveyed had CBSD incidence of <50% and >50% in 2010 and 2012, respectively (Fig. 5).

There were significant difference ($P<0.05$) between the cassava cultivars in both 2012 and 2012 in CBSD incidence. The disease was highest on cultivar Cadri (98.7%) followed by Robero (96.0%), Colocopue (89.3%), Tomo (73.0%) and lowest on cultivar Amwalikampiche (16.7%) in 2010. On the other hand, a low diversity of cassava cultivars was recorded in 2012. Similar to 2010, CBSD incidence was highest on Cadri (100%), but lowest on cultivar Likonde (Table. 3).

Table 2: Incidence and symptom severity of CBSD in three major cassava growing provinces in Mozambique, in 2010 and 2012

Province	2010			2012		
	No. of fields	Incidence (%)	Severity (1-5 scale)	No. of fields	Incidence (%)	Severity (1-5 scale)
Nampula	41	48.3	2.6	44	48.3	2.7
Cabo Delgado	33	23.6	2.4	46	35.4	2.4
Zambezia	41	61.3	2.8	56	82.2	2.9
Mean		**44.4**	**2.6**		**55.3**	**2.7**
P (0.05)		**0.015**	**<0.001**		**<.001**	**0.013**
L.S.D		**31.8**	**0.075**		**12.17**	**0.376**

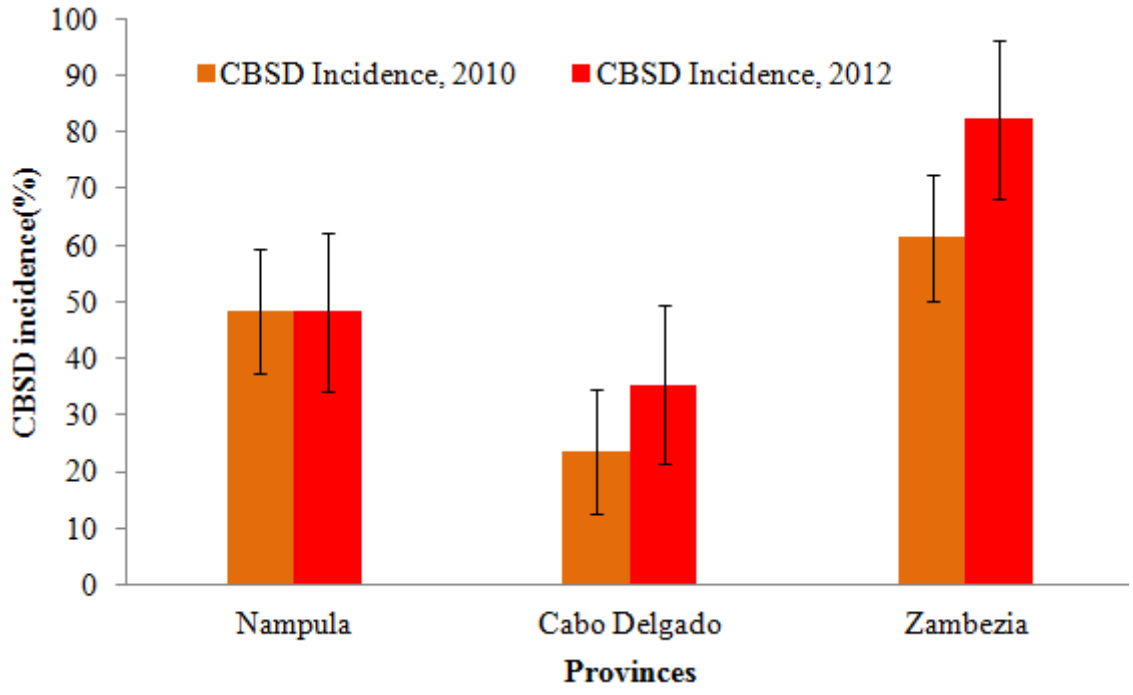

Figure 4: Incidence of CBSD in three major cassava growing provinces in Mozambique, 2010 and 2012

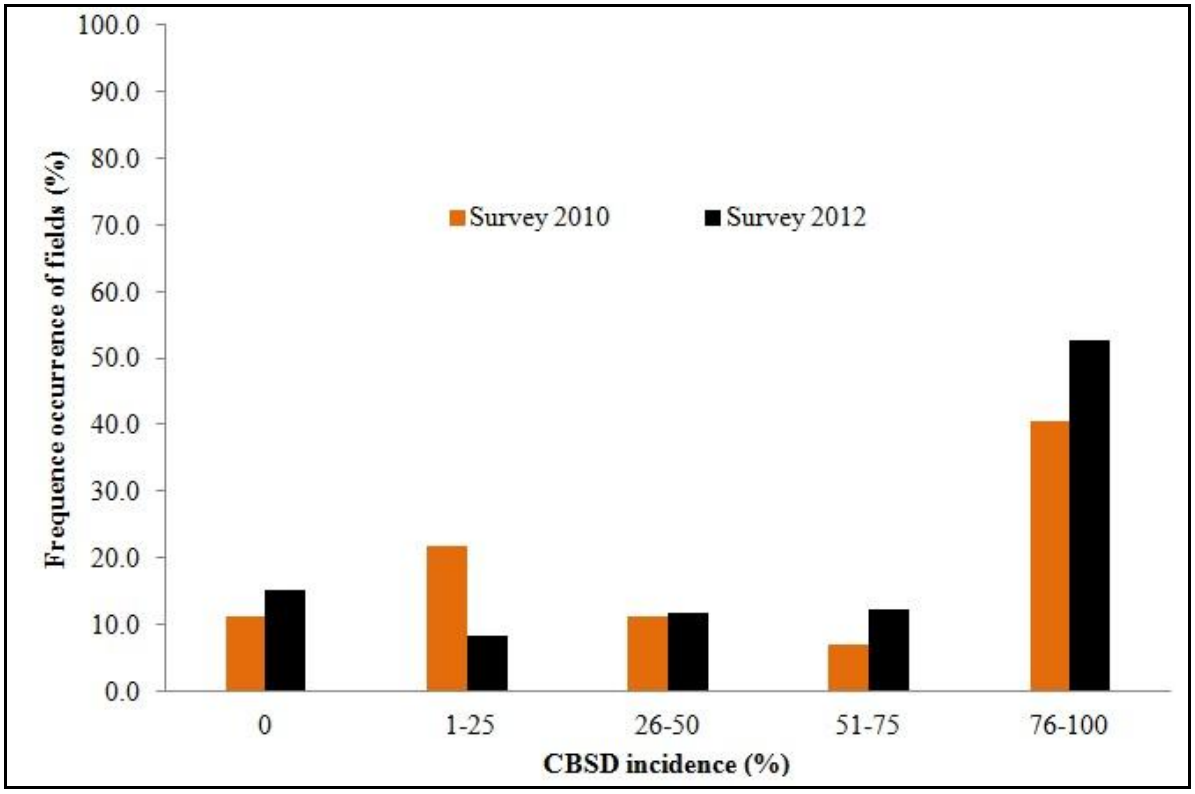

Figure 5: Frequency distribution of cassava fields infected with CBSD incidence (%) in Mozambique in 2010 and 2012

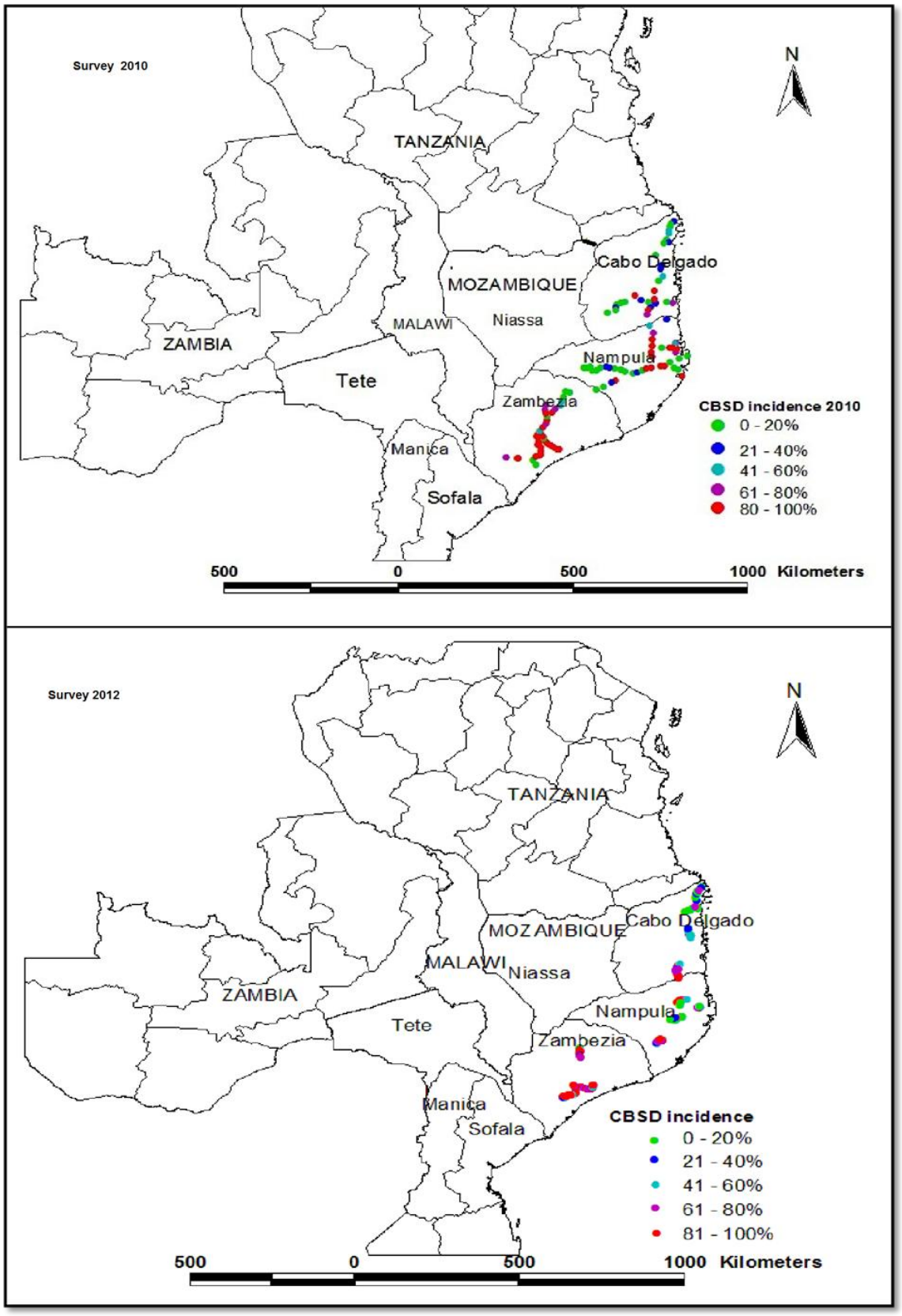

Figure 6: Geographical distribution of CBSD incidence per field in 3 provinces in northern Mozambique, in 2010 and 2012

2.4.3. CBSD symptom severity

Disease symptom severity differed significantly ($P<0.05$) between provinces in both 2010 and 2012. CBSD severity ranged ranged from 2.4 to 2.8 and 2.4 to 2.9 in 2010 and 2012, respectively. In both 2010 and 2012, the symptoms were most severe in Zambezia and least severe in Cabo Delgado (Table 2 & Fig. 7).

Table 3: Incidence and symptom severity of CBSD in most frequently grown cassava cultivars in Mozambique in 2010 and 2012

Cultivar	2010			2012		
	No. of fields (N)	Inc. (%)	Mean sev. (1-5 scale)	No. of fields (N)	Inc. (%)	Mean sev. (1-5 scale)
Amwalikampiche	10	16.7	2.1	0	-	-
Tomo	10	73	2.6	0	-	-
Colocopue	10	89.3	2.7	0	-	-
Cadri	5	98.7	3	12	100.0	2.9
Robero	5	96	2.6	0	-	-
Likonde	5	42.5	2.2	5	0	1.0
Mukudu Moyehiha	5	68.9	2.7	0	-	-
Nachinyaya	5	50	2.1	0	-	-
Angolano	5	64	2.2	0	-	-
Binte massude	0	-	-	5	56.7	3.3
Lipocalane	0	-	-	5	20.0	1.4

Others	5	70	2.3	18	54.1	2.7
Means		**66.9**	**2.5**		**46.16**	**2.26**
P (<0.05)		**0.001**	**0.001**		**0.001**	**0.105**
L.S.D		**34.09**	**1.09**		**68.4**	**1.9**

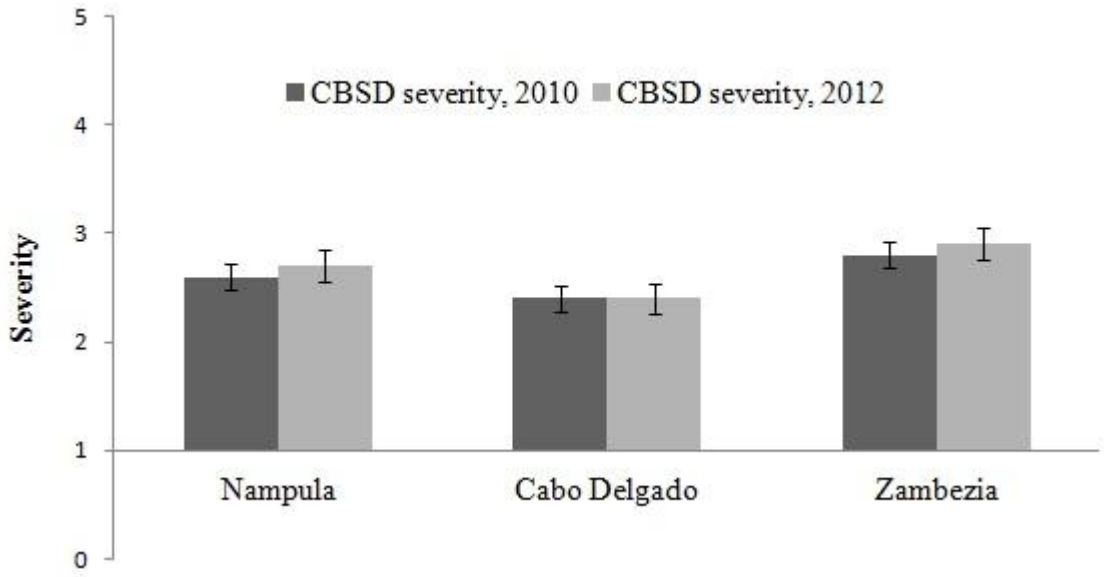

Figure 7: CBSD severity in in three major cassava growing provinces in Mozambique, 2010 and 2012

Analysis of symptom severity of 10 most frequently grown cultivars showed that cultivar Cadri (3.0) had the most severe CBSD symptoms. This was followed by Mukudo moyeviha, Colocopue and Tomo, which had 2.7, 2.7 and 2.6, respectively (Table 3). In 2012, cultivar Binte Massudi (3.3) had the most severe symptoms followed by Cadri (2.9), while no symptoms were observed on cultivar Likonde (Table 3).

2.4.4. PCR amplification of CBSaVs

Using the species specific primer pair CBSDF2 & CBSDR to screen the viruses, CBSV was readily detected in all the CBSD symptomatic samples with the expected size (344 bp) of the partial coat protein (CP). However no UCBSV was amplified in any of the samples (Fig. 8).

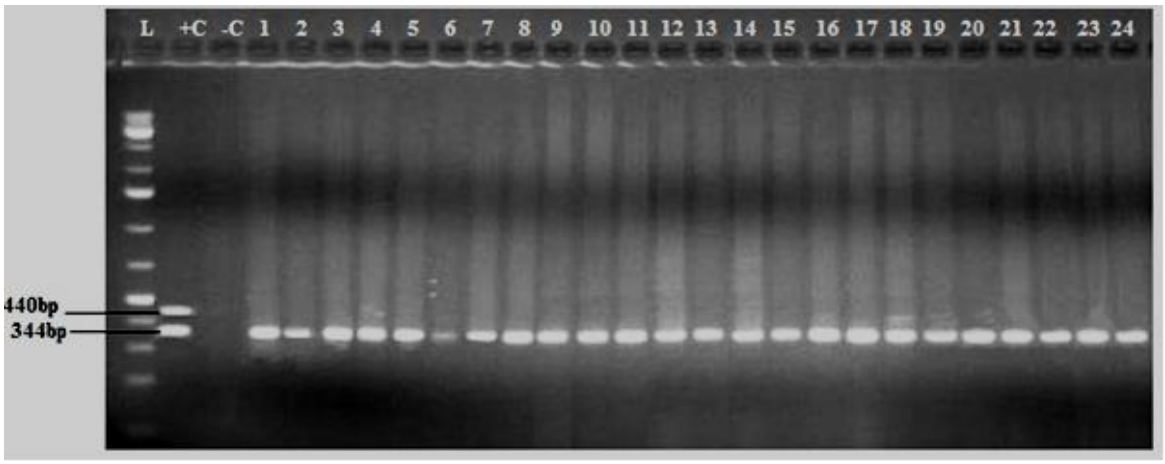

Figure 8: 1% agarose gel of PCR amplified products (344 bp) of a fragment of the CBSV coat protein gene using the specific primer pair: CBSDDF2 and CBSDDR and a 1 Kb plus ladder.

2.5. Discussion

We report here the first comprehensive assessment of CBSD in Mozambique. The disease has been known to occur in the country since the early 1990s and was believed to cause high yield losses (Thresh *et al*. 2002). The study revealed Zambezia to be the most affected province with incidence of 43% in 1998. These findings were in agreements with the ones reported previously (Zacarias *et al*., 2004).

Similar to the earlier findings, Zambezia province was found to have the highest CBSD incidence in both 2010 and 2012. A comparison of the CBSD incidence reported in four consecutive field surveys conducted in 1999, 2003, 2010 and 2012 clearly indicates that the disease situation is worsening. A likely explanation for the increase in the proportion of CBSD affected crops is that farmers may be replanting new fields with disease-affected cuttings. Future studies should investigate the cause of the high disease incidence.

Interestingly, Nampula province had a similar disease situation in 2010 and 2012 in our study. It is not clear why this was so, but this is an area where key activities of the Cassava National Programme (CNP) are based. Nampula was the first province to benefit from improved cassava varieties released by CNP in 2009, which may have contributed to a lower disease incidence compared with Zambezia. It is also important to establish where farmers select planting materials for future use as this could certainly help control the spread of the disease. One of the other suggested reasons for the general spread of CBSD in the northern provinces has been the failure to routinely screen cassava stakes for CBSaVs using reliable diagnostics such as RT-PCR methods. Our study suggests that the combined measures including: efficient virus indexing of the planting materials and vigorous dissemination of new improved varieties with CBSD resistance be routinely done in all the disease affected provinces.

Using field collected disease data on the ten most widely grown cassava cultivars in Mozambique in 2010 and 2012, it appears like there were no improved varieties grown by farmers. The majority of the cultivars assessed showed to be susceptible to CBSD with cultivars such as Cadri recording the highest disease incidence (98.7% in 2010 and 100% in 2012). Amwalikampiche and Likonde, which had relatively low CBSD incidence, are

actually tolerant cultivars that were selected for distribution to farmers by the CNP (INIA-IIAM, 2004). Formal release of improved varieties started only 4 years ago and it will be important to establish how these materials are adopted by the farmers.

Based on the RT-PCR amplification of samples collected in the two surveys only one species, namely CBSV was detected in the CBSD affected areas. We propose that the PCR products are sequenced to confirm the identity of the virus in subsequent studies as it is possible to fail picking up other species such as the UCBSV.

2.6. Conclusion

The results highlight an urgent need to institute control measures to curb further spread of CBSD in Mozambique. This study propose that since CBSD was found to occur in only three out of the six major cassava producing provinces, strict quarantine should be enforced to prevent the introduction of the diseases in the unaffected areas.

Although the development of CBSD-resistant varieties is still in its infancy, options involving accelerated breeding should be considered. A key intervention would be to institute the routinely rigorous and reliable diagnostics for screening of cassava planting material prior to distribution to the farmers. Immediate interventions should begin in Zambezia province, where the current disease incidence is alarmingly over 80%.

CHAPTER THREE

GENETIC DIVERSITY OF CASSAVA BROWN STREAK VIRUSES BASED ON ANALYSIS OF PARTIAL SEQUENCES OF THE COAT PROTEIN GENE IN MOZAMBIQUE

3.1. Abstract

A study was conducted to establish the genetic diversity of of Cassava brown streak virus species in Mozambique. Virus isolates were collected from CBSD-symptomatic leaves and used to extract RNA using a CTAB method. The RNA was amplified using specific RT-PCR primers in the coat protein gene and products sequenced. CBSD-affected cuttings were collected and used to reproduce the field symptoms in cassava and *N. benthamiana*. Results with RT-PCR revealed for the first time the presence of *Cassava brown streak Uganda virus* (UCBSV) in Mozambique. UCBSV occurred in mixed infections with CBSV, with the latter pre-dominating in all the surveyed provinces. UCBSV was restricted in parts of Zambezia province. Phylogenetic analysis of the CBSV sequences revealed two subgroups of CBSV isolates in Mozambique, which diverged by 5.9 to 6.7%. Infectivity assays of CBSaVs inoculated into *N. benthamiana* were conducted successfully, with the virus isolates inducing a variety of CBSD-like symptoms.

3.2. Introduction

Cassava brown streak disease (CBSD) is caused by two positive sense ssRNA viruses (genus *Ipomovirus*, family *Potyviridae*) named *Cassava brown streak virus* (CBSV) and *Uganda* C*assava brown streak virus* (UCBSV) (Mbanzibwa *et al*., 2009a; Winter *et al*., 2010). The two viruses have been confirmed to be transmitted by whitefly (*Bemisia tabaci*) vectors and also spread through planting CBSV-infected planting materials (Maruthi *et al.*, 2005). Transmission by infected cuttings is the main cause of spread in the smallholder cassava fields. CBSV was the virus species first reported to be associated with CBSD in many countries where the disease occurs and is the most predominant. On the other hand, UCBSV is restricted, except in Burundi where it is associated with CBSD (Bigirimana *et al*., 2011).

Molecular analysis of eight partial CP encoding sequences of CBSV isolates from Tanzania and the Lake Victoria basin in Uganda revealed the isolates to be 75.8-77.5% identical at the nucleotide sequence with close similarity to isolates from lowland Tanzania and Mozambique (Mbanzibwa *et al*., 2009a). Incidence of CBSD was shown to be increasing in Zambezia, one of the three provinces reported to be affected by the disease in Mozambique (Chapter 2). Similar to earlier reports by Monger *et al*. (2001a), Mbanzibwa *et al*. (2009a) and Abarshi (2012), CBSV was reported to be the prevalent virus causing CBSD in the country.

CBSD continues to cause devastating effects on cassava crops leading to huge yield loss in Mozambique. It is therefore important to characterise the viruses causing the disease in the country. Genetic diversity will constitute a key element for predicting the disease impact, and devising appropriate and reliable control strategies for disease management. The aim of the

study was to determine the genetic diversity of CBSaVs associated with CBSD in Mozambique.

3.3. Materials and methods

3.3.1. Reproduction of CBSD symptomatic plants

Cassava stem cuttings were collected from plants with diverse CBSD symptoms in farmers' fields in Nampula and Zambezia between June 2010 and June 2012 (Table 4). The symptoms were categorized as follows: (a) foliar+stem+root symptoms, (b) foliar+stem symptoms, (c) without root symptoms, (d) with root symptoms and (e) without aerial symptoms (Table 4).

The cuttings were used to mantain CBSD symptomatic plants in the screen house at IIAM-Nampula Research Station (NRS) for use in screening and and infectivity assays on *N. benthamina* as described by (Goodin *et al.*, 2008).

Table 4: Samples collected in 2010 for virus identification from Nampula and Zambezia provinces and associated plant symptoms

Sample No.	Province	Cassava variety	Latitude	Longitude	Type of symptoms
1	Nampula	Buana	-15.78000000	39.26707872	R, S and L
2	Nampula	Nlapa	-15.82584389	39.24653434	R
3	Nampula	Calamidade	-15.86400000	39.89388889	R, S and L
4	Nampula	Tomo	-15.85158333	40.51158333	R, S and L
5	Nampula	Nacalaça	-14.98855556	39.41138889	R and L
6	Nampula	Nretheta	-14.63572159	40.43544176	R and L
7	Nampula	Fernando po	-14.42509585	39.89565390	R, S and L
8	Nampula	Napriti	-14.94815871	39.93372845	R, S and L
9	Nampula	Caramacha	-15.18886111	39.28447222	R and L
10	Nampula	Nassuruma	-15.07692779	39.74062784	R, S and L
11	Nampula	Murula ohawa	-15.69622222	39.41286111	R, S and L
12	Nampula	Tomo	-15.02952042	39.61659570	R, S and L
13	Zambezia	Robero	-17.35220723	37.05057780	R, S and L
14	Zambezia	Cadri	-17.55609191	36.69858447	R, S and L
15	Zambezia	Paka	-17.57500000	36.65138889	R and L
16	Zambezia	Muriba	-17.50278007	36.90695984	R, S and L
17	Zambezia	Robero	-17.69027778	36.85833333	R, S and L
18	Zambezia	Robero	-17.63886136	36.72178231	R, S and L
19	Zambezia	Cadri	-17.26134606	37.04224971	R and L
20	Zambezia	Adra	-16.93083333	36.95000000	R, S and L
21	Zambezia	Tecnico	-17.54863160	36.79479045	R, S and L
22	Zambezia	Mulezebe	-17.62638889	36.21694444	R, S and L
23	Zambezia	Manite	-17.50278007	36.90695984	R, S and L
24	Zambezia	Fernando interno	-17.22944444	37.12388889	R, S and L

R = necrotic symptoms on the root, S= streak on the stem with different degree and L = appearance of symptoms on the leaves

3.3.2. Mechanical inoculation of CBSV isolates in *N. benthamiana*

Three weeks old plants of *N. benthamiana* were used for the infectivity assays with a total population of 20 plants (15 experimental and 5 control plants). Infectivity tests were done using three CBSV isolates: BO5, BO1 and CO1 with Gene bank accession numbers KC237219, KC237220 and KC237221, respectively. CBSD-symptomatic leaves were obtained from plants with the three CBSV isolates crushed in Carborundum powder to

ensure wounding and mechanical viral inoculation. The sap of the CBSV-infected leaves containg each isolate were rubbed on the leaves of five *N.benthamiana* plants using a soft small brush. All the plants were maintained at 25^{o}C under controlled conditions.

Cassava plants were monitored for symptom development beginning at emergence of first leaves until 9 months at 2 weeks intervals. On the other hand symptoms were recorded at weekly intervals for *N. benthamiana* plants. Symptom severity was recorded as described by Monger *et al*., (2001b) and Bua & Namara (2008).

3.3.3. RNA extraction

Total RNA was extracted from 150 mg of CBSD-affected cassava (24) and *N.benthamiana* (12) leaves, using the modified CTAB protocol (Mbanzibwa *et al.,* 2009a). The yield of RNA was quantified using a Thermo Scientific NanoDrop 2000/2000c (full spectrum UV-Vis) at A260/280 ratio for all samples.

3.3.4. RT-PCR amplification of CBSV partial coat protein gene

Total RNA obtained from 3.3.3 was used to synthesize cDNA in 2 steps using ImProm-II TM reverse transcriptase Kit (Promega, UK) following the manufaturer's instructions. Synthesis was performed in a total of 25 µl as follows description: First step, MASTER MIX 1 consisted of 9 µl of sterilized distilled (sd) water, 1 µl of oligo dT (10 µM) and 4 µl of RNA template. The mixture was incubated by heating at 70^{o}C for 5 minutes and immediately chilled on ice for 2-5 minutes. Step 2, MASTER MIX 2 consisted of 6.5 µl of sd water, 4 µl of 5X RT buffer, 1.0 µl of dNTPs (2.5 µM), 0.5 µl of RNAse inhibitor and 1.0 µl of reverse transcriptase. The reaction was mixed gently and 13 µl aliquotes dispensed into each 12 µl

reaction tube containing MASTER MIX1 making up a total volume of 25 µl. The reverse transcription was performed in the following cycle: 42^{o}C for 60 min, 70^{o}C for 10 minutes, and the resulting cDNA synthesized was used for PCR. The remaining cDNA was stored at -20^{o}C for further analysis.

First, the universal primer pair: CPCBSV1 (CAAACAARDAARAGGCCRTG) and CPCBSV2 (TCGGCDAGRAARTCWATACC) designed to amplify a fragment of 785 bp of the CBSaVs coat protein gene were used. A PCR reaction mix volume of 25 µl consisting of 13.6 µl of sterile de-ionized water, 3.0 µl of 10X PCR buffer+20mM $MgCl_2$, 1.0 µl of primers CPCBSV1/CPCBSV2 (10mM), 2.0 µl of dNTPs, 0.4 µl of Taq polymerase and 4.0 µl of cDNA was used. PCR was conducted in a ICycler Biorad® version 4.006 thermocycler with a hot start at 94°C for 3min, followed by 35 cycles of 94°C (45 sec), 56°C (45 sec) and 72°C (10min) for denaturation, annealing and extension, respectively. PCR products were analyzed by electrophoresis in a 1×TAE buffer on a 2% agarose gel stained with ethidium bromide.

Second, to screen for the virus species, a second PCR was conducted using the 24 and 12 cDNAs obtained from cassava and Nicotiana samples using the primer pairs: CBSDDF2 (5-GCTMGAAATGCYGGRTAYACAA-3) and CBSDDR (5-GGATATGGAGAAAGRKCTCC-3) with expected fragments of the coat protein gene yielding 344 bp (CBSV) and 430-440 bp (UCBSV). The PCR reaction mix of 25 µl consisted of 12.9 µl sterile de-ionized water, 3.0 µl of 10X PCR buffer + 20 mM $MgCl_2$, 1.0 µl of the primers CBSDDF2/CBSDDR (10mM concentration), 0.3 µl of U *Taq* DNA polymeramase, 2.8 µl of dNTPs (2.5 mM) and 4.0 µl of cDNA template. The PCR programme cycling was carried out at: 94°C for 2 min for initial denaturation followed by 35 cycles of 94 °C (30

sec), 51°C (30 sec) and 72 °C (30 sec) for denaturation, annealing and extension, respectively. PCR products were analyzed by electrophoresis in a 1× TAE buffer on a 2% agarose gel, stained with ethidium bromide (0.5 mg/ml).

3.3.5. Sequencing and phylogenetic analysis of CBSaVs isolates

Bands of the expected size on agarose gels were physically excised and purified using a Qiagen gel Purification Kit (QIAGEN, Venlo, the Netherlands) as per the manufacturer's instructions. The purified PCR products were sent to North Caroline State University (NCSU) in the USA for sequencing. Each sequence was edited manually to produce a consensus sequence of 785 bp for each individual isolate using the EditSeq Program in DNAStar. The sequences were aligned with references sequences of CBSV CP available in GenBank using the Clustal W (weighted) (Thompson *et al*. 1994) algorithm available in the MEGA 5.02 software program. The reference CP sequences included: UGUg: 23 [FN434109.1]; UG Wak 39-09 [HM171313.1]; Ug Wak 33-09 [HM171312.1]; Tz Zan 11-08 [HM346960.1]; Tz Zan 7-08 [HM346958.1]; TzMlb 3-07 [FJ039520.1]; TzBsa 4-07 [EU 916832.1]; Mz Namp 1-07[HM346953.1]; MWKar 17:09[HM171319.1]; MWMa 43[HM433933.1] and MWMa 42[FN433932.1].

A phylogenetic tree was generated using maximum likelihood available in MEGA5 software program. For parsimony analysis, bootstrapping (Felsenstein, 1985) was performed with PAUP using the heuristic option for 1000 replication at a 70% confident limit (Swofford 1998). A pairwise comparison of the partial CP nucleotide sequences, expressed as percent nucleotide similarity and divergence between CBSV and UCBSV isolates identified in

cassava in Nampula and Zambezia provinces, was calculated by Clustal algorithm (Thompson *et al*., 1994).

3.4. Results

3.4.1. Reproduction of field symptoms of CBSD under green house conditions

Twenty four of thirty two plants established successfully under green house conditions with typical field CBSD symptoms (Figure. 9). Using the three CBSV isolates: BO5 (accession no. KC237220) and BO1 (accession no. KC237219) from Zambezia and CO1 (KC237221) from Nampula, clear symptoms were reproduced on *N. benthamiana* with: chlorosis which was induced by all three isolates (Fig. 10D); wide necrotic local lesions on the leaves induced by isolate BO5 (Fig. 10A-B); downward leaf curl and blistering (Fig. 10 C); leaf distortion and small local necrosis (Fig. 10 E), growth retardation, systemic infection and death of plant (Fig. 10 a, b, d & e) by all three isolates.

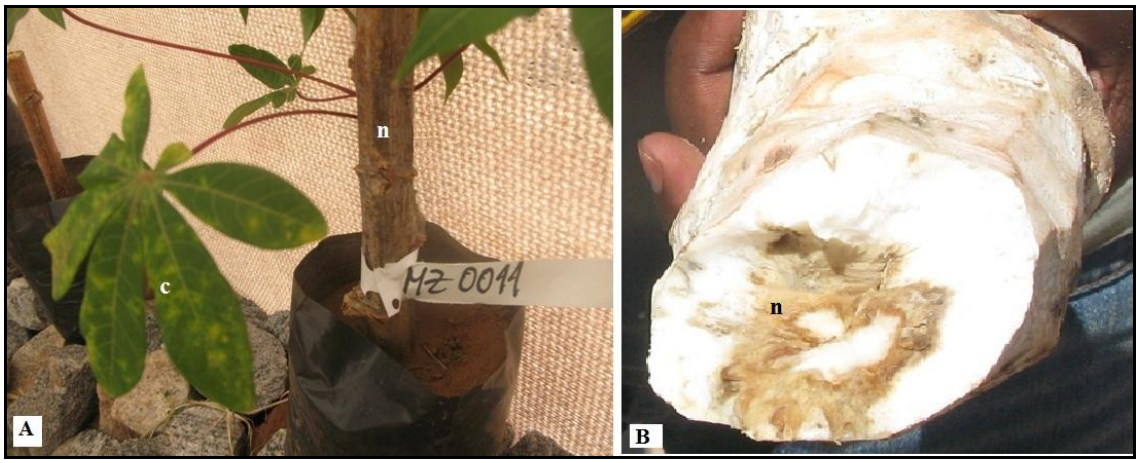

Figure 9: Symptoms produced on (A) leaves – chlorosis(c) and stems – necrosis (n) and (B): roots – necrosis (n) in the field during sampling.

None of the plants used as negative control (uninoculated healthy plants) exhibited leaf symptoms as was expected (Fig. 10 C). This was confirmed by RT-PCR (Fig. 14).

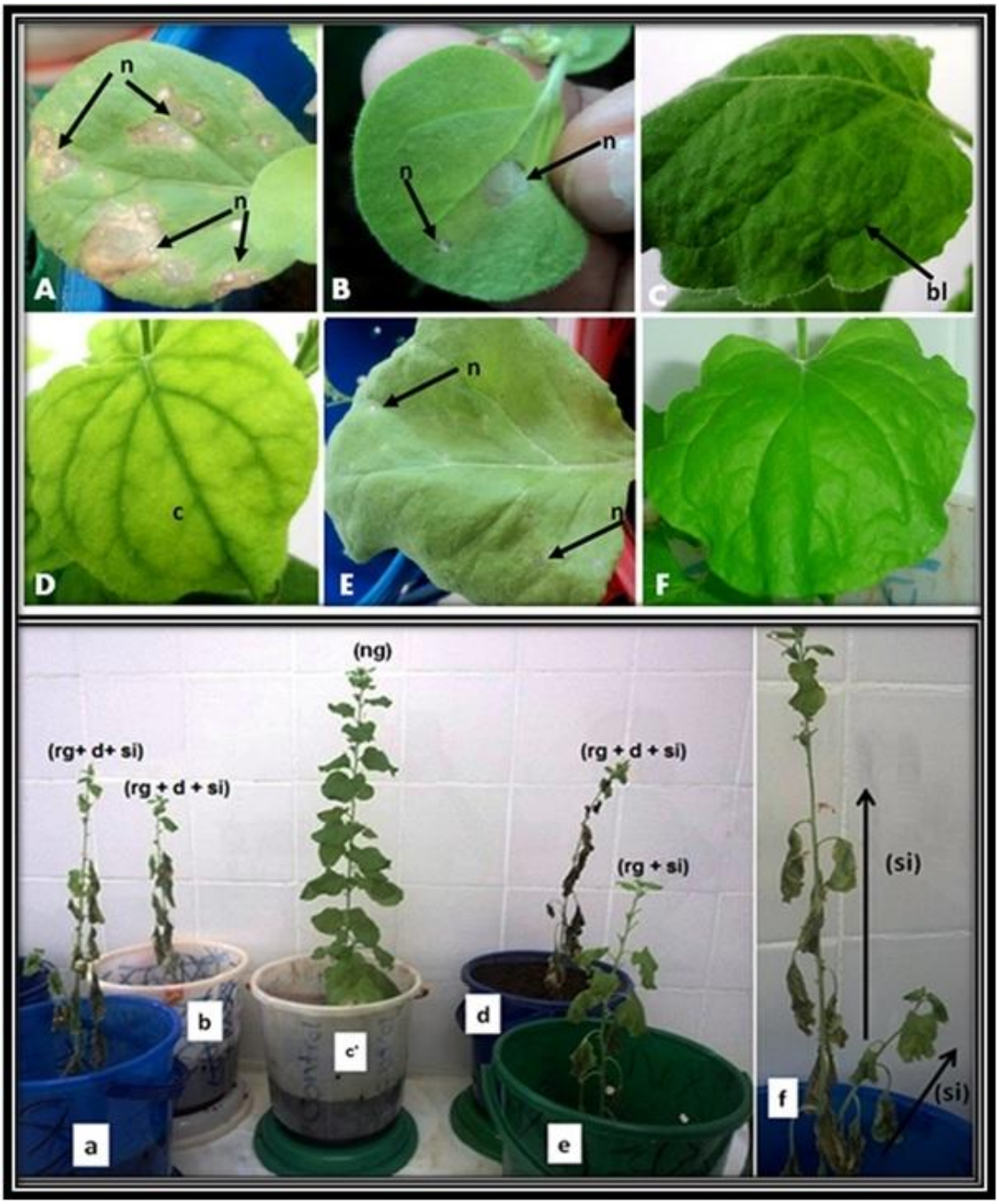

Figure 10: Diversity of CBSD symptoms induced by CBSV isolates on the *Nicotiana benthamiana* at 28 dpi. A-B: necrotic local lesions (n) induced by UCBSV + CBSV; C-E: Symptoms induced by CBSV alone. C: downward leaf curl and blistering (bl); D: general chlorosis (c); E: leaf distortion and local lesions (n); F: healthy uninoculated control; retardation of growth (rg), plant systemic infection (si) resulting in death progressive begin on the basis to the top of plant to up and death (d) of *N. benthamiana* plants isolates infected by CBSV (a, b, d and e); Normal growth (ng) and no symptoms of death in Mock inoculated plants (c').

Variety ‘Fernando interno’ showed CBSD symptoms of spot chlorosis on leaves under screen house conditions (Fig. 11A). When isolate KC237220 was extracted from the leaves of symptomatic ‘Fernando interno’ leaves and inoculated onto the *N. benthamiana,* local necrotic lesions were produced (Fig. 11B & C). The other two virus isolates did not induce these symptoms.

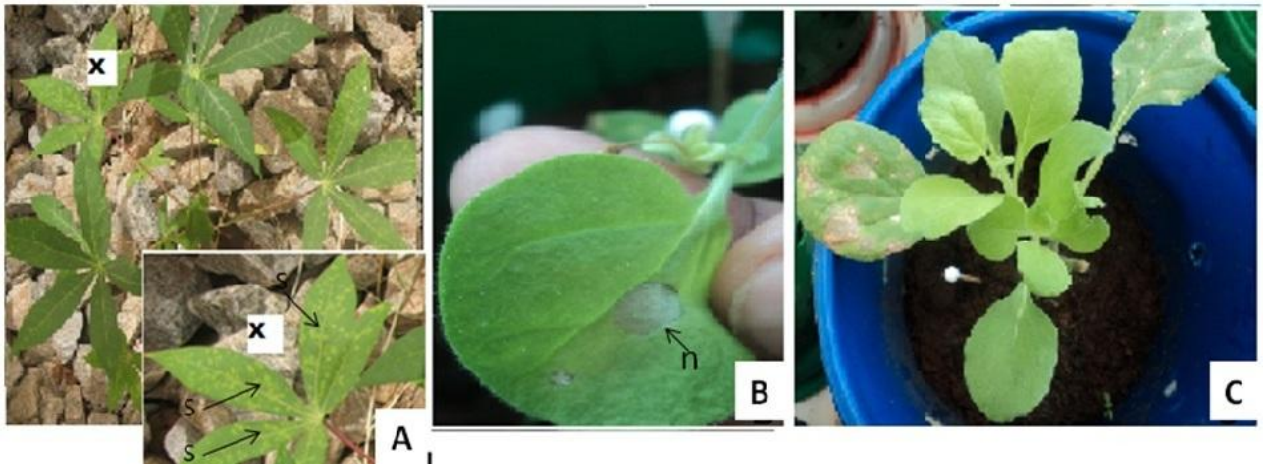

Figure 11: Symptoms produced by variety ‘Fernando Interno’ in screen house conditions: (A) spot chlorosis on leaves; (B) local necrosis (n) on *Nicotiana benthamina* infected by CBSV isolate BO5 (accession no. KC237220) extracted from the leaves of infected variety Fernando Interno (7 dpi) and (C) necrosis at 28 dpi.

3.4.2. RT-PCR analysis of CBSaVs in cassava plants

Identification of cassava brown streak viruses (CBSV and UCBSV) in cassava samples collected in Mozambique was achieved using the universal primer pair: CPCBSV1 & CPCBSV2. CBSaVs were readily detected in all 24 leaf samples with the expected band size (785 bp) of the partial coat protein gene (Fig. 12). However, distinction between the two species was not possible using this primer pair.

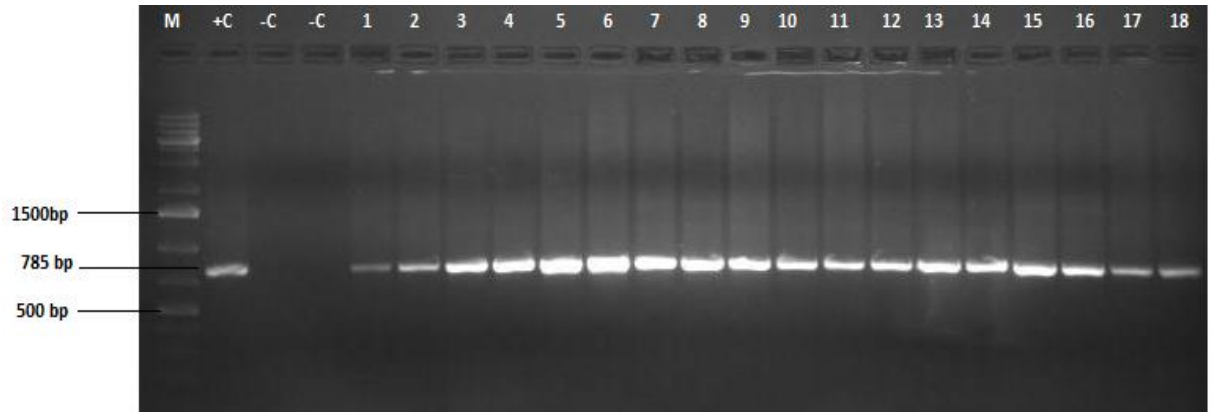

Figure 12: 1% agarose gel of PCR amplified products (785 bp) of CBSV and UCBSV coat protein gene fragment using the universal primer pair CPCBSV1/CPCBSV2. M = ladder (1Kb); +C = Positive Control, -C = Negative control. Lanes 1-18 are the PCR products from cassava infected leaves.

Using the specific primers, CBSDF2 and CBSDR, CBSV was detected in all 24 samples tested; 79.2% of which were single infections (Figure. 13). On the other hand, UCBSV was detected as mixed infections with CBSV in only 20.8% (5/24) of the samples.

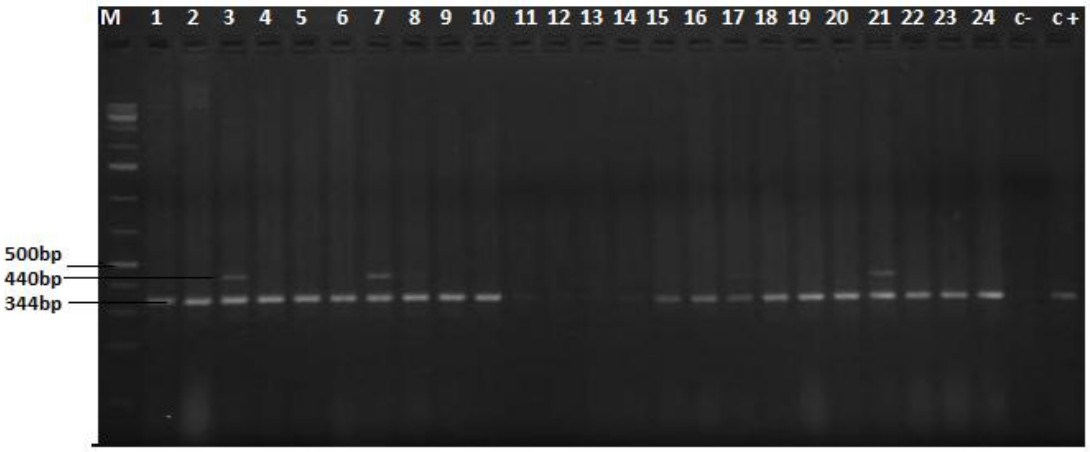

Figure 13: 1% agarose gel of PCR amplified products of CBSV (344 bp) and UCBSV (440 bp) of using the specific primer pair CBSDDF2 and CBSDDR. M = ladder (1Kb); +C = positive Control, -C = negative control. Lanes 1-24 are PCR products from cassava infected leaves.

3.4.3. RT-PCR analysis of CBSaVs in *N.benthamiana* plants

Cassava brown streak virus (CBSV) was detected in nine *N.benthamiana* leaf samples (Fig. 14, 1-6); three of which were co-infections (CBSV+UCBSV) (Fig.4, 7-9). No amplification was obtained for the uninoculated control plants as expected (Fig.14, 10-12).

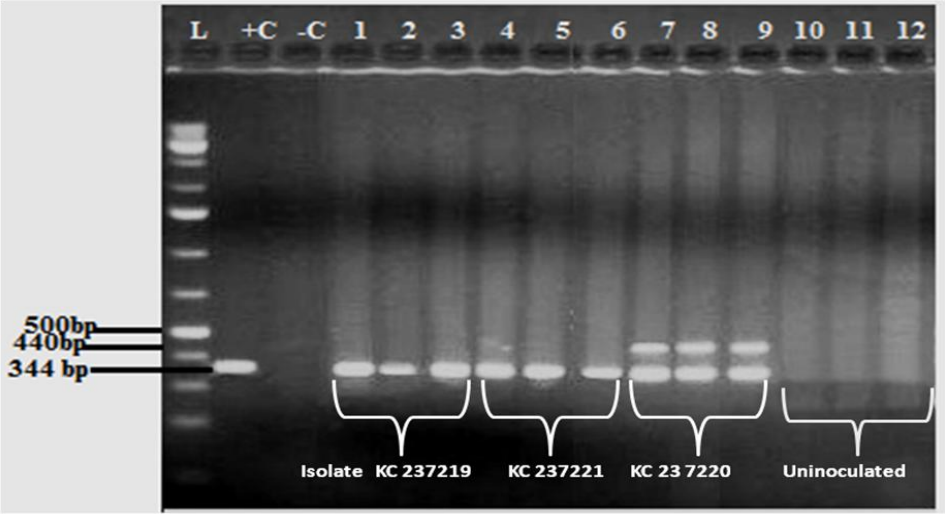

Figure 14: 1% agarose gel of PCR amplified products of CBSV (344 bp) and UCBSV (440 bp) coat protein gene fragment using the specific primer pair: CBSDDF2 and CBSDDR. M = ladder (1Kb); +C = positive Control, -C = negative control. Lanes 1-3 are the PCR products from *N.benthamiana* infected leaf samples with isolate 1; 4-6 are the PCR products from *N.benthamiana* infected leaves samples with isolate 2; 6-9 are the PCR products from *N.benthamiana* infected leaf samples with isolate 3 from 'Fernado interno' cassava cultivar and 10-12 were from *N.benthamiana* uninoculated control plants.

3.4.4. Geographical distribution of cassava brown streak associated viruses in Mozambique

About sixty three percent (12/19) and 36.8% (7/19) of the field-collected samples positive with CBSV occurred in Nampula and Zambezia provinces, respectively. CBSV was the only virus species that occurred in Nampula province and comprised at least 58.3% (7/12) of the Zambezia samples. No single infections of UCBSV were detected in any of the two

provinces. On the other hand, mixed infections of CBSV+UCBSV were detected only in Zambezia province, specifically in Mocuba and Nicoadala districts.

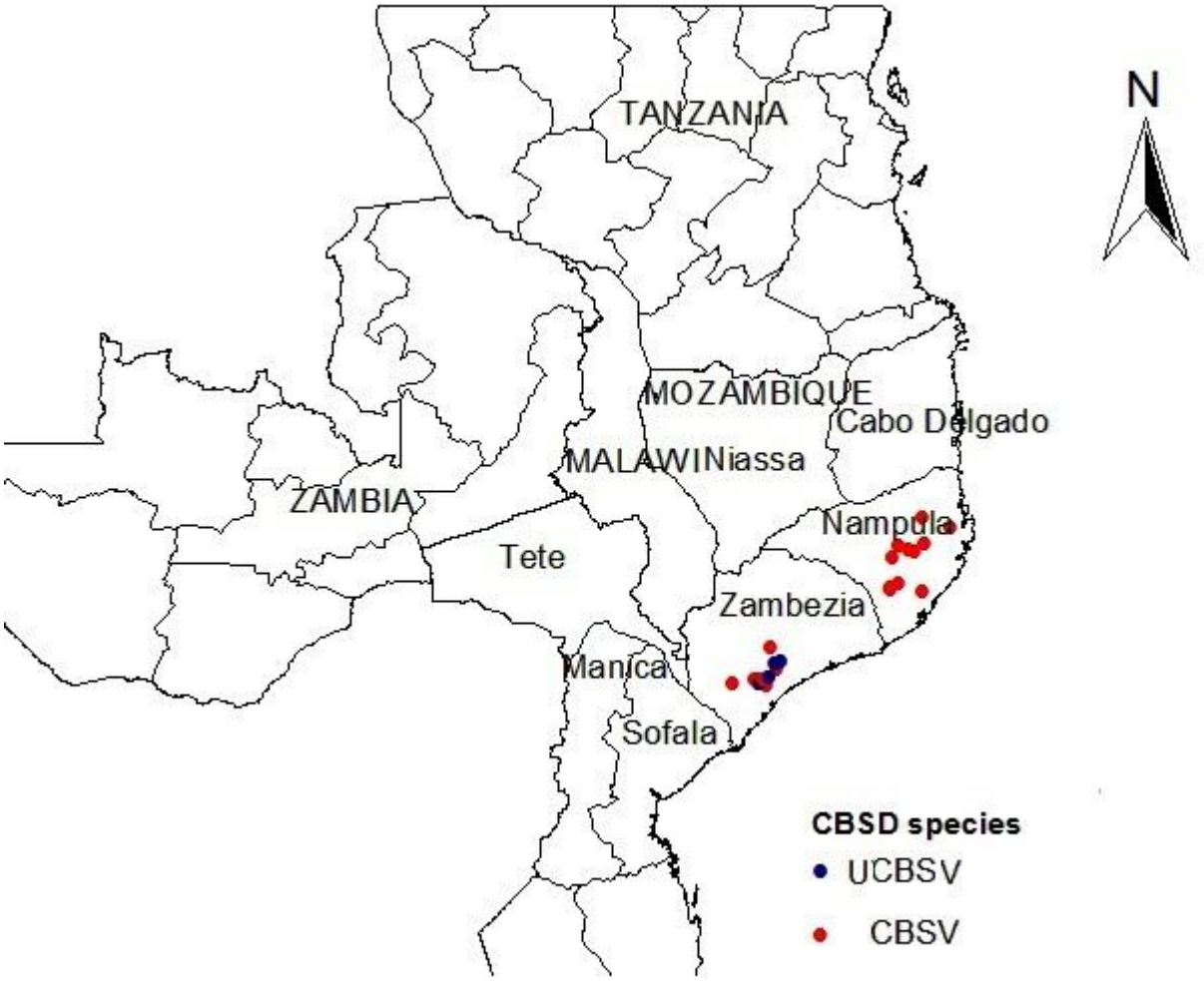

Figure 15: Geographical distribution of cassava brown streak viruses in Mozambique

3.4.5. Phylogenetic analysis of CBSV coat protein gene

Based on BLAST analysis of the nucleotide sequences obtained in the study, all the 8 isolates clustered in phylogroup I, which comprised of CBSV isolates with closest similarity (93.9% - 95.1%) to isolates from Malawi (MW:Kar 17:09), Tanzania (Tz: Zan 7: 08 and Tz :Zan 11: 08), Uganda (UG: Wak 33:09) and Mozambique (Mz: Nmp 1:07). Subgroup Ia isolates

(GO1, BO1, BO5, BO9, CO1, CO9 and EO1) shared a high nucleotide sequence similarity of 98.1%-99.7% among themselves (Table.5). In contrast isolate CO5 shared a nucleotide sequence similarity of 93.3-94.1% with the other 7 isolates (Table.5). None of the sequences clustered in phylogroup II, which is comprised of UCBSV (Fig. 16).

Table 5: A pairwise comparison of the partial CP-encoding nucleotide sequences of virus isolates expressed as percentage nucleotide similarity, and divergence between CBSV and UCBSV isolates from cassava samples from Nampula and Zambezia as calculated by Clustal algorithm.

CBSV isolate		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	GO1	-	98.7	98.1	98.7	97.6	93.3	98.9	98.4	79.1	78.3	93.0	93.9
2	BO1	1.4	-	98.9	99.5	98.4	94.1	99.2	99.7	78.6	78.3	93.9	94.7
3	BO5	1.9	1.1	-	98.9	97.9	94.1	98.7	98.7	79.1	78.9	93.9	94.7
4	BO9	1.4	0.5	1.1	-	98.9	94.1	99.2	99.2	78.6	78.3	93.9	94.7
5	CO1	2.5	1.6	2.2	1.1	-	93.6	98.1	98.1	78.1	77.8	93.3	94.1
6	CO5	7.2	6.3	6.3	6.3	6.9	-	93.9	94.1	78.6	79.7	93.3	94.7
7	CO9	1.1	0.8	1.4	0.8	1.9	6.6	-	98.9	79.1	78.9	93.9	94.7
8	EO1	1.6	0.3	1.4	0.8	1.9	6.3	1.1	-	78.6	78.3	93.6	94.4
9	UG:UG 23	25.1	25.9	25.1	25.9	26.7	25.9	25.1	25.9	-	94.1	79.7	79.9
10	MW:Ma 42	26.4	26.4	25.6	26.4	27.2	24.4	25.6	26.3	6.3	-	79.7	79.7
11	MZ:Namp 1:07	7.5	6.6	6.6	6.6	7.2	7.2	6.6	6.9	24.3	24.4	-	97.9
12	Tz:Zan 7:08	6.6	5.7	5.7	5.7	6.3	5.7	5.7	6.0	23.9	24.4	2.5	-

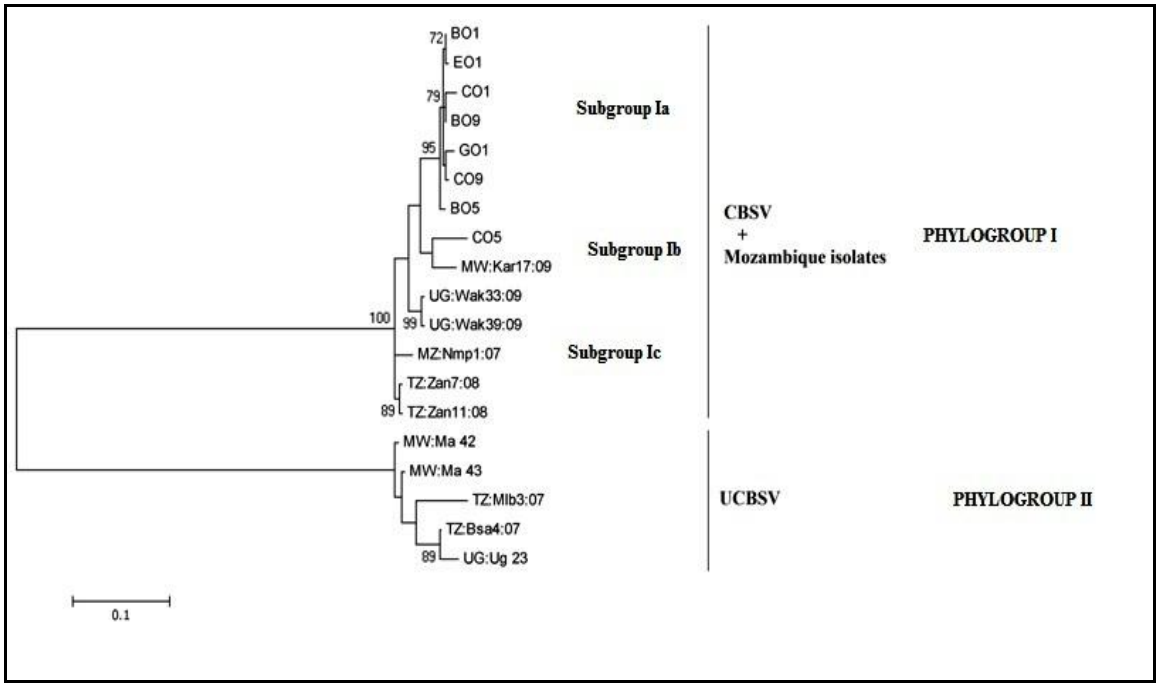

Figure 16: Phylogenetic tree based on partial CP-encoding nucleotide sequences of virus isolates collected in Nampula and Zambezia provinces in Mozambique, generated using maximum likelihood available in MEGA5 software program. CBSV isolates (BO1, BO5, BO9, CO1, CO5, CO9, EO1 and GO1) characterized in this study grouped in subgroup I.

3.5. Discussion

Based on PCR results UCBSV was detected in Mozambique. Earlier, the non-detection of UCBSV in the country was either due to lack of sensitivity by primers or a result of new spread/introductions of the species in the country. There is probably no evidence for new spread, but introductions are more likely through programmes that distributed CBSD-torelant cultivars in the country in the 1990s and early 2000 or exchanging of cassava germplasm by farmers. Earlier studies by other workers (Mbanzibwa, 2009a; Rwesagira, 2009; Abarshi, *et al*., 2012 and Thresh *et al*., 2002) detected only one species (CBSV) to be associated with CBSD of cassava cultivars in Mozambique. Results from this study also support the previous findings; suggesting that CBSV is the most widely occurring virus species infecting cassava in the country.

Based on the partial CP gene sequences of the eight representative CBSV virus isolates characterized in this study, there were two clear sub groups (Ia and Ib), which diverged by 5.9 to 6.7. However, sequencing of the full CP sequence, or even other open reading frames of CBSV isolates in Mozambique may reveal even more diversity. The seven isolates of subgroup Ia shared high nucleotide sequence similarity of 98.1-99.7%, except for isolate CO5, which was clearly genetically distinguishable from the other 7 isolates and shared less nucleotide sequence similarity (93.3-94.1%). The sequence similarity observed in this study among the CBSV isolates was higher when compared of those reported by Mbanzibwa *et al* (2011b) which the variation among the CBSV isolates was wider (79.3-95.5%).

Geographic distribution of two viruses causing CBSD showed the CBSV to predominate in Nampula and UCBSV to occur in mixed infections in Zambezia province only. UCBSV was found in Mocuba and Nicoadala districts. The samples used in the study were obtained from only two provinces (Nampula and Zambezia). Wider sampling should be done to test more cassava cultivars for CBSaVs with multiple primers and sequencing.

Infectivity assays to *N.benthamiana* plants were successfully conducted using mechanical inoculation. RT-PCR confirmed the presence of CBSaVs in all the leaves collected from the infected *N.benthamiana* plants. The diversity of symptoms (necrotic local lesion, downward leaf curl and blistering, leaf distortion and local necrosis, growth retardation, systemic infection and death of plant) elicited on *N.benthamiana* in the current study suggests that the isolates may have different virulence. Similar symptoms were observed by Monger *et al.* (2001b) on *N. benthamina,* with three CBSVs isolates from Tanzania on *N.occidentalis* (Mbanzibwa *et al*., 2009a), on *N. benthamiana* (Abarshi *et al*., 2012) and on five Nicotiana species (Bua and Namara, 2009). More research is needed to understand clearly how these two distinct CBSV species interact and what the biological and epidemiological implications could be. Knowledge of the viruses causing CBSD is key in developing mitigation measures. This requires efficient and robust diagnostic tools to detect and differentiate the causal viruses.

3.6. Conclusion

The results based on the RT-PCR and phylogenetic analysis showed that both CBSV and UCBSV virus species are found in Mozambique. This indicates that CBSaVs diversity in Mozambique has changed since the study by Thresh *et al,* (2002). The presence of UCBSV

is evidence that virus diversity has changed in Mozambique, although it is not known when UCBSV could have been introduced. However, surveys including extensive collection of CBSV isolates, and characterization of full-length CP sequences, are still required to undertand the diversity of CBSaVs.

The present study provided further evidence that CBSV isolates are efficiently transmitted to *N.benthamiana* plants, and the study yielded more information on diversity of symptoms phenotypes and death were induced in all *N. benthamina* plants inoculated which indicates that CBSV can cause severe and even fatal symptoms. These results suggest that the CBSV could have wider host range, therefore, more research is needed to identify other hosts of CBSD, which constitute reservoirs for enhancement of new infection in cultivated areas.

4. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Based on the current findings, it is clear that CBSD incidence is increasing in Mozambique, with Zambezia as the most affected province. More than 50% of the fields assessed between 2010 and 2012 had a high proportion of disease infections. This shows that there are limited efforts to control the disease. There is urgent need to institute control measures for the disease such as: restricted movement of CBSD-affected material between districts and provinces, rigorous screening of cassava planting material, introduction of CBSD tolerant and resistant cultivars, such as Amwali kampiche and Likonde and awareness campaigns. The cultivar Cadri, Robero, which are all susceptible, should be avoided.

Using field collected disease data on several varieties, it was observed that the majority of cultivars grown by farmers showed to be susceptible. Efforts to multiply the new improved

varieties and distribution for the farmers are required to reduce the impact of this devastating disease. Immediate intervention of multiplication and dissemination of new improved cassava varieties should begin in Zambezia province, where the disease dramatically increases over 2 years (2010 and 2012).

It is reported in this study, for the first time, that UCBSV species occurs in Mozambique, as prior studies done by other workers had reported only CBSV species to be associated with CBSD. It is confirmed the wide persistence of CBSV specie in the northern country, while UCBSV was found restricted in some areas of Zambezia province. The presence of isolates of both viruses could have implication on breeding programs in Mozambique. A more comprehensive surveys including new areas were cassava is growing are still required to screen the CBSaVs and in fact where they spreading.

Based on the phylogenetic analysis, it was clear that CBSV isolates in Mozambique are heterogeneous at the isolate level, and were clustered in two subgroups, this serves to demonstrate that genetic diversity of cassava brown streak viruses is large, and predict that wider screening of full length DNA sequences would be likely to reveal a higher degree of genetic diversities among the CBSaVs isolates in Mozambique.

CBSV isolates were efficiently transmitted to *N.benthamiana* and diversity of symptoms caused by CBSV species were observed. Cassava brown streak viruses could have other alternative host range, however there is scanty information on the alternative hosts of the viruses. Only few reports exists (Bock, 1994; Mbanzibwa *et al.*, 2011a and Mware *et al*., 2010). Such studies have not been conducted in Mozambique, and therefore, more research is needed to identify other eventual hosts of CBSV as the strategy to control virus disease.

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