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ASPECTS OF THE EPIDEMIOLOGY OF CASSAVA BROWN STREAK VIRUS DISEASE IN TANZANIA

THESIS

BY

GRATION MUTASHOBERWA RWEGASIRA

Thesis Submitted to the Faculty of Science of University of the Witwatersrand in Fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

2009

DECLARATION

I, Gration Mutashoberwa Rwegasira, do hereby declare to the Faculty of Science of University of the Witwatersrand, Johannesburg, that the content of this thesis is my own original work, and has never been submitted, nor concurrently being submitted for any degree award in any other University.



14TH AUGUST, 2009

_____ _____

Gration Mutashoberwa Rwegasira **Date**

School of Molecular and Cell Biology,

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ABSTRACT

In this study, the field diagnostic survey supplemented with CBSV detection by RT-PCR using virus coat protein gene-specific primers, revealed the presence of CBSD throughout the cassava growing areas of Tanzania. Sequence data of 43 CBSV isolates suggested considerable diversity among although the majority cladded together.

CBSV transmission was achieved through cutting tools with 22 % efficiency using test plants of susceptible cassava cv. Albert. Sap transmission of the virus had 54 % efficiency in cv. Mreteta. Grafting of CBSV-free susceptible scions onto CBSV-infected rootstocks was the most efficient transmission technique with up to 100 % recipient plants cv. Albert infected within 4-weeks after inoculation. The virus was not transmitted through infected root debris or seeds.

Three major types of foliage and root symptoms for CBSD were apparent. The foliage symptoms included veinal chlorosis, chlorotic blotches and chlorotic spots. The internal root symptoms were brown necrotic mass, necrotic specks and chalky necrosis in the root cortex. The nature of occurrence of these symptoms was also elucidated. The tender leaves, youngest symptomatic leaves and non-necrotic portions of the root cortex were suitable for detection of the virus by RT-PCR. CBSV-free plants could not be regenerated from CBSV-infected cuttings.

Foliage chloroses were found to be suggestive but not an absolute indication of CBSV infection. Only 67 % of tested field samples were both symptomatic and infected by the virus; 22 % were CBSV-free despite being symptomatic; 7 % were infected but symptomless; 4 % were CBSV-free and symptomless.

CBSD incidences and severities in different cultivars varied between seasons and were dependent on the inherent cultivar characteristics. Foliage and stem CBSD incidences and severities were significantly ($P < 0.001$) related to the individual cultivars. The effect of plant age

on CBSD incidences and severities was also significant ($P < 0.05$). Low temperature (26^0C day and 18^0C night temperature) was critical and lethal to CBSD-affected plants. More than 50 % and 100 % of test plants died within 30 and 50 days respectively, post-exposure to this temperature. Moisture stress led to deaths of all CBSD-affected test plants within 90 days.

DEDICATION

To my wife, Rozalia and my children, Musimiseki, Hubert and Mujuni who endured loneliness during my long absence from home but showed patience and love. Also to my late grandmother Ms. Thereza Karoli who set my roots to school life but did not live long enough to see me complete this great work.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Cassava (*Manihot esculenta* Crantz) production in Africa has for long been affected by virus diseases (Calvert and Thresh, 2002). Among diseases reported in Tanzania is cassava brown streak disease (CBSD). The disease is caused by *Cassava brown streak virus* (CBSV) (Family: *Potyviridae*; Genus: *Ipomovirus*) (ICTV, 2005; Monger *et al.*, 2001a). CBSD has been known in cassava production systems in Tanzania since early 1930s (Storey, 1936), but became more damaging in areas away from the coast in the early 1990s (Hillocks and Thresh, 1998). It is known to occur in the coastal zone of the country extending from the northern border with Kenya to the southern border with Mozambique (Nichols, 1950a). Yield loss per plant due to CBSD (in terms of fresh tubers) is estimated at above 70 % (Hillocks *et al*., 2001). The etiology of CBSD has been partially described (Monger *et al.*, 2001a; Storey, 1936). Unfortunately, there has been limited progress on its epidemiology.

CBSD was for long time regarded as a low altitude disease usually occurring below 1000 metres above sea level (masl) (Nichols, 1950a). However, the disease was recently reported to occur in fields located at mid-altitude of 1,185 masl in Uganda (Alicai *et al.*, 2007; EARRNET, 2005). This suggested a change in the CBSD pathosystem. In addition to the presence of CBSD at unexpectedly high altitudes in Uganda, the strains of the causal virus, CBSV, were reportedly different from the Mozambican and Tanzanian isolates (Monger *et al.*, 2001b). The current status and geographical distribution of CBSD in Tanzania is not understood. The sequence variation

among CBSV isolates and symptom variability also suggests the possible existence of diverse strains in Tanzania.

Planting of infected cuttings, mechanical and vector-based transmissions are the three mechanisms through which CBSV is known to spread (Legg and Hillocks, 2003). Transmission efficiency of the whitefly, *Bemisia tabaci* was reportedly low *ca* 2 % (Maruthi *et al.*, 2005). The non-vector mechanisms which are suspected to contribute largely to CBSV transmission and spread are not well explored. Early researchers on CBSD (Bock and Guthrie, 1976; Lister, 1959; Storey, 1936) reported on grafting and sap inoculation as the major means through which CBSV is transmitted experimentally. However the infectivity and efficiency of these mechanisms were not determined. Other non-vector mechanisms through which the virus may be transmitted have never been explored. Some plant viruses have been reported to spread through farmers' cultural practices such as infected farm working tools, handling of infected plants, seeds, vegetative propagation and infected plant debris left on farm (Agrios, 2005). Mechanical transmission of viruses has been reported in potato spindle tuber viroids and potato virus X (Manzer and Merrian, 1961).

Following CBSV infection, symptoms occurred on shoot and storage roots of the affected plants (Nichols, 1950a). Most published reports on CBSD are based solely on observable symptoms some of which may not be caused by CBSV infection. Diverse symptoms were recently observed on CBSD-affected plants, some of which have never been reported previously. The relationship between CBSV infection and symptoms expression has never been established. Limited knowledge on the distribution of CBSV in various organs of the infected plants makes it

difficult to delineate the most appropriate choice of sample for CBSV detection. Consequently, the knowledge gaps complicate the diagnosis of the disease.

The severity of CBSD on infected plants was reported to vary depending on the cultivar and environment (Jennings, 1960). The magnitude of disease severity was reflected on the level of damage sustained by the affected plants and consequently the yield loss. Nichols, (1950a) suggested altitude, temperature and variety response as main determinants of disease severity in CBSD-affected plants. However, the critical levels of temperature and the response of susceptible cultivars to the disease with time have never been determined. Other environmental factors affecting the severity of the disease are not well explored. The knowledge on these parameters is vital in understanding the nature of the interactions between the virus, the infected plants and the environment.

The knowledge gap on these epidemiological parameters is a major impediment in understanding CBSD and designing appropriate management techniques. Unless the nature of interactions between CBSV, the susceptible cassava cultivars and the environment is explored and understood, CBSD will continue to cause economic loss and devastation to the cassava farming community in Tanzania.

1. 2 Literature review

1. 2. 1 Epidemiology: the origin and definition

Etymologically, the term ‘epidemiology’ derives from the Greek terms, *epi* = upon; *demos* = people; *logos* = study. It literally means ‘the study of what is upon people (Wikipedia,

2008). Hippocrates, a Greek physician is believed to be the inventor of ‘epidemiology’ when he used the term ‘epidemic’ to describe diseases that are seen at some times but not others (Anon, 2008a). Although it was originally applied to human populations, the term is also used in studies of animal and plant populations (Nutter Jr., 1999).

Epidemiology has been defined in various ways depending on the author’s perception and the field of application. In the Merriam-Webster dictionary (2008), epidemiology is defined as a branch of medical science that deals with the incidence, distribution and control of disease in a population. Matthews (1991) defined epidemiology as the study and investigation of the distribution and causes of the disease. Other definitions for epidemiology includes the following; The study of patterns, causes and control of disease (Agrios, 1997); study of the causes, distribution and control of disease in a population (American Heritage, 2006); and, the study of incidence, distribution and control of disease (Anon, 1998). The definition given by Schwabe *et al.,* (1977) that, epidemiology is the study of disease in populations, is the one which seems widely accepted and has been adopted by FAO. Whatever definition used and regardless of the field of study, the key terms are; **the causes**, **distribution pattern** and **control of the disease**.

Epidemiology may be descriptive or analytical (Anon, 2004). Descriptive epidemiology refers to the study of the disease in a population (Ellis-Christensen, 2008). It presents incidence and severity data on the disease in relation to the host, place and time and in terms of their consequences to the general population. It characterizes the burden of the disease in a population. Analytical epidemiology refers to the study of determinants of the disease (Anon, 2007). These include causative factors, host characteristics and the environment that influence the risk of

developing or contacting the disease. It characterizes the risk factors for disease in a population. Considering all these concepts and in the context of this study, epidemiology may realistically be defined as “the study in which plant health problems are detected, investigated and analyzed such that management strategies can be designed and implemented”.

In an attempt to understand the epidemiology of cassava brown streak disease, a basic understanding of cassava (the host) plant, the causal agent of the disease (*Cassava brown streak virus*), the mechanism through which the virus is transmitted to susceptible plants and the factors affecting the occurrence and perpetuation of the disease are vital. The current study explores the epidemiology of cassava brown streak disease in Tanzania.

1. 2. 2 Origin and geographical distribution of cassava

Cassava, *Manihot esculenta* Crantz (Euphorbiaceae) formerly known us *Manihot utilissima* Pohl, is a crop with world-wide distribution across the tropics. It is among the leading food crops of the world ranked fourth among major staples (Nassar, 2002). It is drought-resistant and tolerates poor soil fertility. Vries *et al.*, (1976) described cassava as the most efficient producer of carbohydrates and energy among all the food crops. Documented archeological indices estimate that the domestication of the crop began 5000-7000 BC in the Amazon (Allem, 2002; Gibbons, 1990; Pohl, 1827). The anthropological evidence reported by early writers (Rogers, 1963) suggests that the crop’s origin and first domestication was in Brazil. However, lack of concrete data to justify the origin of the crop triggered controversy and led to contradictory speculation and beliefs (Table 1. 1).

Table 1. 1 Suggested countries or regions where the first domestication of cassava took place

Brazil	Pohl, 1827
Brazil	Mueller, 1874
Eastern Tropical Brazil	de Candolle, 1884
Brazil	Pax, 1910
Peru	Cook, 1925
Brazil	Lanjouw, 1932
Northern Amazonia	Schmidt, 1951
Brazil, central Paraguay	Vavilov, 1951
Venezuelan savannah	Sauer, 1952
South America	Anderson, 1954
Peru or Mexico	Rogers, 1963
Brazil	Jennings, 1963
Southern Mexico, Guatemala, Honduras	Rogers, 1965; Rogers and Appan, 1970
Eastern Venezuela	Reichel-Dolmatoff, 1965
Peru	Lanning, 1967
Northern Amazonia	Schwerin, 1970
North America	Lathrap, 1970
Amazonia	Rogers, 1972
Central America and North-eastern Brazil	Spath, 1973
Amazonia	Parseglove, 1976
Meso-America	Schultes, 1979
Brazilian states of Goi?s, MatoGrosso and Rondônia	Allem, 1997
Brazilian states of Mato Grosso and Rondônia	Olsen and Schaal, 1998; 1999.

As quoted by Allem CA, 2002. The Origin and Taxonomy of Cassava In; Hillocks RJ, Thresh JM and Bellotti AC, (Eds), 2002. Cassava: Biology, Production and Utilization; Chapter one, Pg 9.

While taxonomists consider modern cassava (*M. esculenta* Crantz *ssp esculenta*) to have evolved from several progenitors of the wild relatives in the geological past (Hillocks *et al.*, 2002; Heiser, 1990; Rogers, 1963, 1965; Sauer, 1994), molecular phylogeneticists reject the belief. Olsen and Schaal (1999) reported the high level of noncoding sequence variation in cassava and its wild relatives, based on the proof derived from the single copy nuclear gene glyceraldehydes 3-phosphate, dehydrogenase (G3pdh). Despite the different opinions, two facts exist about cassava, which most scientists agree; i) cassava originated from South America (Hillocks *et al.*, 2002), and ii) modern cassava derives from a number of intraspecific hybridizations (Nassar, 2002).

1. 2. 3 Cassava in Africa

Intercontinental movement of cassava to Africa took place between the 15th and 16th centuries (Hillocks *et al.*, 2002a; Jones, 1959) and later on in the 18th century. It was introduced by Portuguese seafarers as they sailed to explore Africa and the rest of the world (Jones, 1959). Two different points of introductions are documented. The first introduction is believed to be in West Africa (Jones, 1959; Hillocks, 2002) where later on, it was grown in the River Congo Delta and Fernando Po in the Gulf of Benin (Hillocks *et al.*, 2002a). This first introduction had limited spread to the rest of Africa. The second introduction was through Reunion Island in 1736 from where it spread to Zanzibar and the rest of East Africa (Purseglove, 1968). It was this second introduction which is believed to have led to the spread of cassava across sub-Saharan Africa. During the 20th century the crop was promoted by colonialists as a food security crop in their

respective colonies. A thorough review on the introduction and spread of cassava across Africa is well documented (Hillocks *et al.*, 2002a).

1. 2. 4 Economic importance of cassava

The multiple uses of cassava make it one of the economically valuable crop around the world. The crop is grown for human food particularly in African countries where in many areas the production of cereal-based food crops is affected by drought and poor soil fertility. The leaves and shoots obtained from the crop are often an ultimate source of proteins and vitamins to the resource-poor families in Africa. In South and Central America, cassava is usually an animal fodder for a wide range of domesticated animals such as poultry, pigs and cattle (Nassar, 2002). The processing of the crop into various forms of industrial products makes it a dependable source of income (Robertson, 1987). Commercialization of the crop ranges from small to large scale (Sarma and Kunchai, 1991). At small scale (household) level, cassava roots and leaves are sold in raw form for food and confectioneries (Henry *et al.,* 1998). Large scale use of various cassava-based products, particularly in the European Community (EC) in early 1980s triggered the commercialization of cassava as an internationally traded commodity (ESCB, 2000). Increased importation of cassava chips and pellet from Asian and African countries into EC was recorded, although the quantities declined towards the late 1990s (FAOSTAT, 2008). The quantity of cassava products exported regionally and internationally (ESCB, 2000), justifies the importance of the crop to the economy of the small and large-scale producers.

Nutritionally, cassava roots provide more than 30 % of the dietary starch consumed in Africa (Okigbo, 2000). In addition to the high carbohydrate content (74 - 85% of its total storage

root dry weight), it contains varying amounts of vitamin C (ascorbic acid), vitamin A (carotenes), iron, zinc, calcium, potassium and proteins, that accounts for its popularity (Baguma and Kawuki, 2006).

In industries, cassava is a vital source of starch, used in paper, plywood, textile industries and pharmaceuticals. Starch is directly used as a smothering agent in industrial production of papers and sizing of threads in textile industries (Chulavatnatol, 2001). Glue made out of starch is used in the plywood and paper-box industries. Domestically starch is useful in soup thickening, baby foods and manufacturing of sweeteners (Hershey *et al.,* 2000). While the notable sweeteners such as glucose, fructose and sorbitol are derived from starch hydrolysis, monosodium glutamate (MSG) is a product of microbial fermentation of starch (Chulavatnatol, 2001). Other uses of starch include production of ethanol and biodegradable plastics (Westby, 2002).

In most African communities, the crop is mainly grown for food with limited use as animal fodder and other industrial products. More than 90 % of the total production across the continent is used as food and less than 10 % is used for feed and other industrial uses (Nweke, 2004).

Recently, cassava was recognized as a crop for peace. In African countries ravaged in civil conflicts such as Angola, Burundi, DRC, Liberia, Mozambique, Sierra Leone and Uganda, the cultivation of annual crops is often handicapped. In these areas, new hopes for peoples' lives are mostly based on cassava (Clark, 2006; Dowden, 2007; Pearce, 2005). Displaced groups of people during the war also survive on long-abandoned cassava fields. The long-term storage of

cassava roots in the ground ensures continued availability of food source in time of food shortage. Moreover, post-conflict retreats of refugees have been aided by the presence of cassava that was abandoned pre-conflict (LWF, 2004). Being a vegetatively propagated crop, such plantings also serve as a ready supply of planting material during rehabilitation following conflict or drought.

1. 2. 5 Worldwide production of cassava

Global production of cassava is particularly concentrated in Africa, Asia and Southern and Central America. Africa leads in total production and area under cassava, but Asia has the highest return per unit area cultivated (Figures 1. 1a & 1. 1b), (FAOSTAT, 2008). In productivity terms, Asia leads Africa, Southern and Central America. Hillocks *et al*., (2002) estimated that, the world annual production of cassava starchy roots reaches more than 158 billion tons used for human consumption (58 %), animal food (22 %), and other uses (20 %). The major component of produced cassava is used as fodder and raw material for industry in Asia and Southern America.

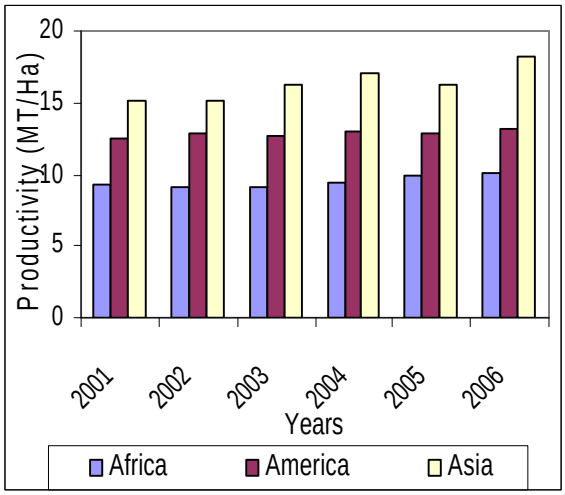


Figure 1. 1a Cassava production per area

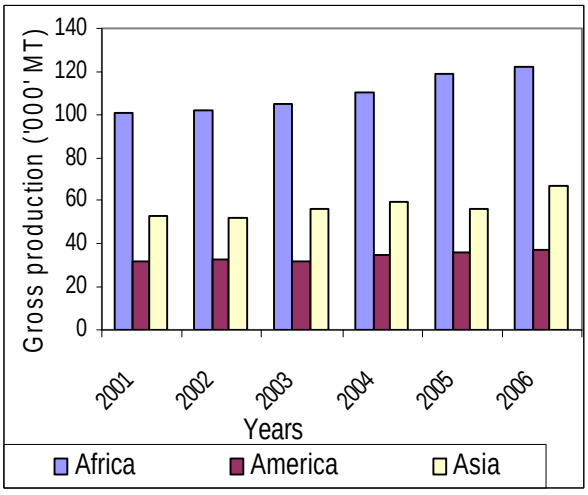


Figure 1. 1b Gross cassava production

In Africa, cassava is grown in 40 countries of which Angola, Benin, Democratic Republic of Congo, Ghana, Madagascar, Malawi, Mozambique, Nigeria, Tanzania and Uganda are the ten leading producers (FAOSTAT, 2008). Recent production data for the crop in the ten major producing countries in Africa are documented (Table 1. 2).

Table 1. 2 Annual production of cassava in ten lead countries in Africa (Million tonnes)

Countries	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**
Angola	5.	6.	6.	8.	8.	8.
Benin	2.	2.	3.	2.	2.	2.
Congo-DRC	15.	14.	14.	14.	14.	14.
Ghana	8.	9.	10.	9.	9.	9.
Madagascar	2.	2.	1.	1.	2.	2.
Malawi	3.	1.	1.	2.	2.	2.
Mozambique	5.	5.	6.	6.	11.	11.
Nigeria	32.	34.	3(	38.	41.	45.
Tanzania	6.	6.	5.	6.	7.	(
Uganda	5.	5.	5.	5.	5.	4.
Total	88.	89.	92.	97.	105.	108.
Total for Africa	98.	100.	103.	108.	113.	117.
Total for World	18:	18!	19:	20:	20!	22:

Source: FAO Statistic Division, 2008, http://faostat.fao.org

The inherent ability of cassava to adapt to diverse agro-ecological conditions, ranging from heterogeneous soils to erratic weather, has influenced its wide cultivation in sub-Saharan Africa (Okigbo, 2000). The crop is most important in coastal areas and in the farming systems of the humid forest regions, where the productivity of grain crops is reduced by poor soil. Poor soils, foliage pests and diseases curtail the productivity of cereals and storage is difficult. The high yield potential realized in cassava makes it a viable alternative to grain crops, particularly in areas where population pressures have led to tradeoffs between food quality and quantity.

1. 2. 6 Production constraints of cassava

The generally low yields of cassava in Africa are attributed to abiotic and biotic constraints (Legg et *al.,* 2006). The major abiotic constraints are poor soils and unpredictable weather (Anon, 2002). The soils in most of the cassava producing countries are generally poor, sandy, and frequently mineral deficient (Hillocks *et al.,* 2002a). Erratic rainfall and prolonged dry spells with no reliable irrigation systems in place, compound the productivity problems.

Biotic constraints primarily comprise a range of pests and diseases. Arthropod pests including cassava green mite (*Mononychellus tanajoa* Bonder), cassava mealybug (*Phenaccocus manihoti* Matile-Ferrero) and whitefly (*Bemisia tabaci* Gennadius) seriously damages the crop affecting the final yield. While *M. tanajoa* and *P. manihoti* mainly causes direct physical damage, *B. tabaci* is important in inflicting direct damage and also by vectoring viruses. Cassava bacterial blight (CBB) (*Xanthomonas axonopodis* f.sp. *manihoti*) is the most important non-virus disease (Legg and Thresh, 2003) affecting the crop. Cassava mosaic disease (CMD), caused by cassava mosaic geminiviruses (CMGs) (Family *Geminiviridae*; genus *Begomovirus*) (Hong *et al.,* 1993), and cassava brown streak disease (CBSD) caused by *Cassava brown streak virus*

(CBSV) (Monger *et al.,* 2001a), are currently the major threats to the crop's health and productivity.

1. 2. 7 History of Cassava Brown Streak Disease

Cassava brown streak (CBSD) was first reported in Tanzania (Storey, 1936) more than seventy years ago. Since the first report, the disease continued to affect local cultivars most of which were susceptible. The damage associated with the disease caught the attention of researchers in colonial Tanganyika, at Amani Research Station in early 1930s and led to a resistance breeding programme (Storey, 1939).

The detailed work on the unusual symptoms of brown streak (Storey, 1936; 1939) led to an official report of cassava brown streak disease as a problem at Amani, Tanga. The report was followed by a series of investigation on the cause and nature of occurrence of CBSD, which led to a thorough description of the disease symptoms (Nichols, 1950a). Studies on mechanism of spread and management of the disease through resistant cultivars (Jennings, 1957; Nichols, 1950b; Storey and Doughty, 1951) were also conducted. Extensive breeding work mainly concentrated on a search for field-resistant materials from local and internationally acquired germplasm, collected from around the world (Jennings, 1957; Storey and Doughty, 1951). Complete lack of resistance among the collected stock, led to a shift in attention to cross breeding from which resistance to CBSD was achieved. The cultivars, *Aipin valenca* (obtained from Belgian Congo) and *Macaxeira aipin* (obtained from Brazil) were the source of first CBSD-resistant progeny (Jennings, 1957).

Causal agent of Cassava Brown Streak Disease

Capitalising on Storey's (1936; 1939) assumption that the infection agent is likely to be a virus, Lister (1959) reported successful mechanical transmission of the virus to herbaceous indicator plants, *Nicotiana debneyi* Domin. and *Nicotiana benthamiana* Domin. Isolates from infected indicator plants were used effectively to infect susceptible cassava. Advanced studies by Kitajima and Costa, (1964) described the elongate virus-like particles that were detected in CBSD-symptomatic plants using electron micrograph. Bock and Guthrie (1976) confirmed the Lister (1959) findings on mechanical transmission of CBSV. A limitation of these studies was that, they were based on the development of visual symptoms on the respective test plants, which does not identify a specific candidate virus. The fact that cassava test plants used in transmission study were not pre-tested against CBSV due to lack of robust detection techniques, also triggers suspicion of latent infection.

Lennon *et al.* (1986) reported the isolation of elongate filamentous particles 650-690 nm long in *N. benthamiana* inoculated with sap obtained from a cassava brown streak diseased plant. A very low concentration of virus particles was noted. The particle length of 650 nm was morphologically similar to carlaviruses, hence the suggestion that CBSV belonged to the *Carlavirus* genus. Further work on affected plants led to the description of two CBSD-associated viruses as a *Carlavirus* and a *Potyvirus* (Brunt *et al.*, 1990). This caused confusion and difficulties in delineating the actual genus and family to which CBSV belongs. Later work by Harrison *et al.,* (1995) highlighted the presence of 'pin-wheel' inclusions typical of potyviruses in extracts from CBSD-affected plants. The suspicion that potyviruses could be involved due to pin-wheel inclusion was repeatedly supported (Lecoq *et al*., 2000). Molecular studies by Monger

et al., (2001a) confirmed that CBSV was a *Potyvirus* and provided no evidence that a *Carlavirus* was involved.

Renewed concerns on Cassava Brown Streak Disease

There was renewed concern about CBSD in 1987 when a severely affected cassava plant with obvious CBSD leaf symptoms was observed at a location between Kibaha and Morogoro in Tanzania (Thresh, 2003). This triggered the active involvement of scientists in attempts to determine the magnitude of the problem. Detailed studies on the aetiology and epidemiology of CBSD (Hillocks and Thresh, 1998) were prioritized. A series of surveys were conducted in most countries reportedly affected during the early years of CBSD. Assessed countries were; Malawi in 1993 (Hillocks *et al.*, 1996; Sweetmore, 1994); Tanzania in 1993 and 1994 (Legg and Raya, 1998); Mozambique in 1999 (Hillocks *et al.*, 2002b); and Kenya in 2000 (Munga and Thresh, 2002). The disease transmission tests conducted at Kibaha, Tanzania (Hillocks *et al.*, 2001) to identify the potential vectors had limited success. Maruthi *et al*., (2005) reported successful transmission of CBSV by *B. tabaci,* albeit at low efficiency, *ca* 2 % using 20 adult insects per test plant (2 %).

1. 2. 8 Etiology of Cassava brown streak disease

The etiology of Cassava brown streak disease (CBSD) is not well understood (Legg and Hillocks, 2003). Although there has been some progress in describing the morphology, the chemical and physical properties of the virus (ICTVdB Management, 2006) the available information is inadequate. Recent publication of the CBSV full-sequenced genome (Mbanzibwa *et al.*, 2009) has minimized the knowledge gap on the CBSV genomic properties and delineated

the constituted proteins and some of their functions. This recent development is a major breakthrough in designing CBSV management strategies through genetically engineered resistance.

Taxonomy and morphology

According to the International Committee on Taxonomy of Viruses (ICTV, 2005), CBSV is an approved virus species of the genus *Ipomovirus* in the family *Potyviridae*. Other Ipomoviruses includes; *Sweet potato mild mottle virus* (SPMMV), *Cucumber vein yellowing virus* (CVYV) and *Squash vein yellowing virus* (SqVYV) (Adams *et al,* 2005, Lecoq *et al.*, 2000). The complete genomes of Ipomoviruses are positive-sense, single-stranded linear ssRNA with varying genome size (Anon, 2008b). SPMM is 10.8kb (Colinet *et al*., 1998), CVYV is 9.7 kb (Janssen *et al.,* 2005) and SqVYV is 9.8 kb (Li *et al*., 2008). The full genome size for CBSV is reportedly 9.1 kb (Mbanzibwa *et al.,* 2009; Winter *et al.*, 2008).

The CBSV virion consists of a capsid which is not enveloped and has an elongated helical symmetry (Lecoq *et al.*, 2000). It has filamentous, usually flexuous rods (Figure 1. 2a) with a clear modal length of 750 nm. The viral genome is monopartite with only one particle size of linear, positive-sense, single-stranded RNA (ssRNA). It encodes structural and non-structural proteins (ICTV, 2005).

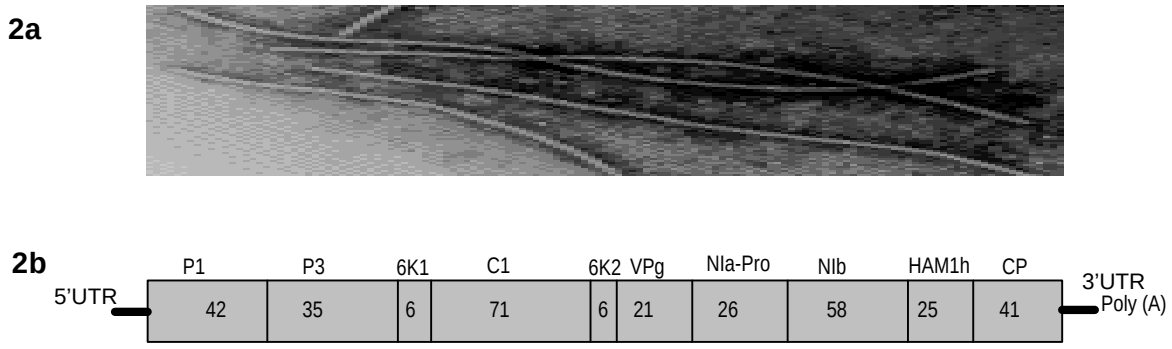


Figure 1: 2a Particle morphology of CBSV (Courtesy: Lennon *et al.*, 1986); and **2b** Schematic presentation of CBSV genome (Courtesy: Mbanzibwa *et al.,* 2009). The particles (2a) are flexuous filamentous rods that encapsidate single positive-stranded RNA molecules of CBSV. The genome structure (2b) contains P1 and P3 proteins, lacks HC-Pro and has Ham1 protein and other proteins that are common in CVYV and SqVYV as indicated above. The boxes separated by lines indicate the putative cleavage sites of the polyprotein.

CBSV genome comprises a typical potyviral polyprotein but lacks a sequence coding for a putative protein HC-Pro (Mbanzibwa *et al.,* 2009; Winter *et al.,* 2008). P1 is the first protein located upstream of the P3 protein (Mbanzibwa *et al.*, 2009; Winter *et al.*, 2008). In the 3'-terminus, the 58K Nlb protein is followed by a stretch of 226 amino acids coding for a Ham1 like protein upstream of the 41K putative coat protein. The current view on the CBSV genome (Mbanzibwa *et al.*, 2009; Winter *et al.*, 2008) suggests a divergence from the Adams (2008) report that the genome structure for *Potyviridae* is conserved throughout the family. Divergence from the viral *Potyviridae* genome has also been reported in other Ipomoviruses such as CVYV (Janssen *et al.*, 2005) and SqVYV (Li *et al*., 2008).

Transmission of CBSV

Transmission of CBSV to susceptible plants was reported to occur vegetatively by grafts and mechanically by sap (Storey, 1936; Lister, 1959; Bock and Guthrie, 1976). Storey (1939) suspected the involvement of a vector, most likely *B. tabaci*, in transmitting the virus. However, several attempt to transmit CBSV through the suspected vectors including aphids, *Myzus persicae* Sulz. (Lennon *et al*., 1986); and whiteflies, *B. tabaci* and *B. afer* (Bock, 1994) were not successful. Despite the failure, speculation on whitefly involvement persisted. Hillocks (2003) speculated that vector abundance and probably the distribution of alternative hosts were the cause of the localized occurrence of the disease along the coastal zone. Robertson (1987) reported that the correspondence of CBSD and whitefly numbers in most fields in East Africa supports the speculation that vectors are involved.

Unfortunately, the reported relationship between CBSD occurrences and whitefly number did not delineate the species involved. While Bock (1994) suspected *B. afer* as the most likely vector for CBSV, the species was reported to be less abundant at the East African coast (Legg and Raya, 1998) compared to *B. tabaci*. In contrast, Munthali (1992) reported the widespread occurrence of *B. afer* in all CBSD-affected areas in Malawi. This confusion persisted until subsequent transmission experiments (Maruthi *et al.,* 2005) confirmed *B. tabaci* as a vector for CBSV although at low efficiency *ca* 2 % using 20 adult whiteflies per test plant.

Virus-Vector Interaction

Most plant viruses depend on vectors for plant-to-plant spread (Ng and Falk, 2006). Approximately 80 % of plant viruses depend on hemipteran arthropods for transmission (Holn,

2007). The piercing-sucking mouth parts of hemipterans such as aphids, whitefly and leaf hoppers, facilitate efficient extraction of plant sap as well as transmission of plant viruses. The interactions between virus and vector for transmission is very specific (Ng and Falk, 2006), and sometimes mediated through capsid and helper components (Ammar *et al.*, 1994).

Early studies on virus transmission by vectors (Watson and Roberts, 1939) indicated the requirement for optimum times for the virus-vector interaction to occur. The length of feeding period required for the vector to acquire the virus (acquisition access period-AAP) and the time to subsequently transmit the virus (inoculation access period-IAP) have led to three different categories of vector-transmitted viruses (Ng and Falk, 2006). When considered alongside the pathway assumed by the respective viruses in their vectors, the three categories are; non-persistent, stylet-borne (take few minutes to hours of feeding), semi-persistent, foregut- borne (few hours to days) and persistent, circulative (days to months). Nault (1997) described a fourth category under the name 'propagative'. This is a circulative form of virus transmission which rarely occurs. In this category, the virus passes to the vector's progeny.

The interaction between CBSV and its whitefly vector, *B. tabaci* is semi-persistent. Maruthi *et al.,* (2005) reported a 48 h requirement on AAP and IAP for successful transmission of the virus. The semi-persistent interaction between whitefly and the virus was also reported in CVYV (Harpaz and Cohen, 1965), a close relative of CBSV. Specific studies on CBSV describing the nature of interaction with its whitefly vector are lacking. However, the involvement of a capsid protein (CP) as reported in vector-based transmission of CVYV (Janssen *et al.*, 2005) could be a feature of CBSV.

Virus-Vector-Host Interaction

B. tabaci transmits many viruses around the world (Morales, 2007). Whiteflies are the second most efficient vectors after aphids, among arthropods (Madden *et al.*, 2000). The preference of *B. tabaci* for cassava to other field crops in the hot-humid tropics makes it an ideal vector for the viruses infecting cassava, including CBSV. The assumed mode of CBSV transmission to cassava is similar to that described by Pirone (1981) in which the virus is retained in the foregut of *B. tabaci* and later introduced into new plants by an ejection-ingestion mechanism. It involves continuous feeding by the whitefly upon phloem to acquire the virus, such that the virus remains in the vector for up to a few days. Viruses transmitted semi-persistently exhibit strong phloem tropism, typical of potyviruses (Ng and Falk, 2006).

In host plants, CBSV is believed to be transmitted systemically through phloem tissue and more extensively through xylem tissues. The movement through plasmodesmata aids the cell-to-cell movement of the virus (Matthews, 2002). The CP has also been implicated in the hosts' cell-to-cell movement and long distance transport of the virus (Dolja *et al.*, 1994).

Virus-Host-Environment Interactions

Successful transmission and infection with CBSV of susceptible plants largely depends on prevailing temperature. According to Maruthi *et al.*, (2005), the successful transmission of CBSV by *B. tabaci* was only achieved at a temperature of $25 \pm 2\ ^{0}C$. Temperature has also been reported to influence symptoms expression in CBSV-infected plants (Nichols, 1950a, 1950b; Storey, 1936). Low temperature augments the effects of the disease to the extent that highly

intolerant clones may even be killed after infection (Nichols 1950b). In contrast, moderate to low disease severity was recorded when high temperature prevailed (Nichols, 1946).

Altitude is another important factor considered to affect the transmission of CBSV. Nichols (1946) indicated that CBSD was not found at altitudes above 1050 masl. The limited abundance of the suspected vector *B. tabaci* and poor survival of CBSV-infected plants were suggested as reasons for the absence of the disease at cooler altitudes. As such, the absence of CBSD-affected plants, limits the amount of the available CBSV inocula. The low whitefly population reduces chances for encountering CBSV-infected plants which leads to the limited transmission of the virus. Considering the reports that CBSV transmission is only possible at elevated temperatures (Maruthi *et al.*, 2005), the altitudinal influence on CBSV transmission could be an indirect one through temperature.

1. 2. 9 Host range, distribution and epidemiology of CBSD

Knowledge of the host range of the causal virus, the nature of occurrence and mechanism of spread to new areas as well as epidemiological factors are vital attributes in understanding the epidemiology of a disease (Matthews, 2002).

Host range

The absence of CBSD in South America and Asia suggests that the disease is endemic to East Africa (Storey, 1939). Thus, the existence of wild alternative host plants in the region was considered most likely. Documented evidence indicated the natural occurrence of the disease in tree cassava, *Manihot glaziovii* (Storey, 1936, 1939). Moreover, the sap transmission

demonstrated during early studies of CBSD (Bock, 1994; Bock and Guthrie, 1976; Lister, 1959) implicated species of three plant families, *Euphorbiaceae*, *Solanaceae* and *Amaranthaceae* as hosts to the virus, those of the first two families being more important. CBSV infection of *N. benthamiana, N. clevelandii* Gray and *Petunia hybrida* (Hook) Vilm. triggered systemic induction of irregular chlorotic vein banding whereas in *N. debneyi*, necrotic lesions developed (Bock, 1994). In *P. hybrida*, the infection with CBSV also caused stunting and necrosis (Lister, 1959). In *Datura stramonium* (L.) Torr. and *D. ferox* L, the virus caused systemic chlorotic stippling mixed with vein banding. During the winter season in UK glass house, CBSV-infected *D. stramonium* developed local necrotic lesions. Bock, (1994) also implicated *Solanum nigrum* L. and *Chenopodium quinoa* Willd. as alternative hosts to the virus.

Occurrence and spread into new areas

CBSD was first reported in the foothills of the Eastern Usambara Mountains in Tanganyika (Tanzania) in the vicinity of Amani Agricultural Station (Storey, 1936). The disease was believed to have spread from the wild plants to cultivated cassava. It was reportedly widespread in the coastal cassava growing areas of Tanganyika (Tanzania), by late 1930s (Storey, 1939). Fifteen years later, all East African cassava growing coastal areas extending from the north-eastern border of Kenya to the Mozambique border in the south were known to be affected (Nichols, 1950a). In Uganda, the disease was first reported at Bukalasa Experimental Station in 1945. It was assumed to have been introduced through infected cassava planting materials sent from Amani in 1934 (Jameson, 1964). CBSD occurrence in Uganda triggered an intensive roguing campaign in 1948/49 (Nichols, 1950a; Thresh *et al.*, 1994). This appeared to be successful and CBSD was not recorded in Uganda until in 1994 when it was observed near

Entebbe in Southern-Central Uganda (Thresh *et al.,* 1994). Recent reports indicated the occurence of the disease to the interior parts of Uganda (EARRNET, 2005; Alicai *et al.,* 2007) at about 1200 masl.

A series of surveys conducted in the early 1990s confirmed Nichols' (1950a) report on the presence of CBSD in Malawi, Mozambique and Tanzania (Hillocks *et al*., 1996; Legg and Raya, 1998; Munga and Thresh, 2002; Sweetmore, 1994; Thresh and Mbwana, 1998). The disease distribution reports in these countries revealed a concentrated occurrence of the disease along the eastern coast with high incidences in most of the cultivated cassava varieties (Hillocks and Thresh, 1998). In southern Tanzania, the disease was more common at an altitude below 300 masl, less common between 300m and 500 masl and rare at altitudes above 500 masl, (Hillocks *et al.,* 1999).

Temporal and spatial spread of CBSV

Natural spread of CBSD in originally uninfected stocks of cassava has been reported (Hillocks, 1999; Kanju *et al.*, 2003). In recent field experiments which involved CBSD-free, CMD-resistant clones imported to Tanzania from Colombia (CIAT), most plants soon succumbed to CBSV (E. Kanju, pers. comm.). Observations on field experiments repeatedly indicated that, most exotic cultivars became infected by CBSV (Jennings, 1957; Storey, 1939).

Hillocks *et al.,* (1999) reported on the sporadic occurrence of CBSD in local cultivars. However, the factors involved were not elucidated. Two phenomena are suspected to have been involved. The first is latently infected cuttings, which were unknowingly used as planting

materials. Secondly, the planting materials were free from CBSV but new infections were whitefly-borne. The low efficiency of the virus vector, *B. tabaci ca* 2 % (Maruthi *et al.,* 2005) resulted in only a few plants infected. The reasoning however, does not exclude the possible existence of other mechanisms of CBSV spread. In contrast, the large scale spread of CBSV was possibly through farmers and breeders' exchange of planting materials, both locally and internationally (E. Kanju, pers. comm.).

Epidemiological implication of CBSD

The observed occurrence of CBSD in originally uninfected stock suggests the occurrence of CBSV inocula in or around farmers' fields and the potential to culminate into an epidemic whenever conditions are favourable and susceptible cassava is grown. The possibility for a changed CBSD pathosystem (favourable environment for CBSV infections and subsequent development of CBSD symptoms) is also evident due to the reported occurrence of the disease in areas previously perceived unfavourable. Thus, the CBSD could no longer be restricted to low altitude. In addition, the spread of CBSV could be facilitated through tolerant varieties due to limited expression and seasonality of the disease symptoms (Jennings, 1957). In a situation that robust and affordable CBSV detection techniques are lacking, the disease may spread faster than would otherwise be expected.

1. 2. 10 Symptoms associated with CBSD

The name cassava brown streak derives from the brown lesions which sometimes appear on the young green stems of diseased plants (Storey, 1936). Nichols (1950a) made the first comprehensive description of CBSD symptoms. In the most susceptible cultivars, infection with

CBSV is manifested through varied symptoms that are expressed on either or both shoots and roots of the diseased plants (Nichols, 1950a; Hillocks and Jennings, 2003). However, the nature of symptom expression depends upon the environmental conditions, sensitivity of the cultivar and growth stage of the crop relative to the time at which infection occur (Hillocks and Thresh, 1998). Hillocks *et al*., (1996), described variable symptom of CBSD and attempted to establish the relationship between shoot and root symptoms which varied among cultivars. Despite some variations, the common symptoms that are considered to truly reflect the infection with CBSV were described (Hillocks and Jennings, 2003). CBSD symptoms are often manifested in the leaf, stem, fruit and roots; and may be expressed in only one, two or more organs either separately or simultaneously (Nichols, 1950; Hillocks, 1997; Hillocks and Jennings, 2003).

Leaf symptoms

Two types of leaf symptoms have been described (Nichols, 1950a; Hillocks, 1997). Firstly, a leaf chlorosis which starts along the margins of secondary veins expanding to cover the tertiary veins and may finally produce chlorotic blotches. Secondly, a chlorosis which develops in roughly circular patches between the main veins and may affect much of the lamina. The pale shade of yellow is a dominant colour usually related to the second type of symptom (Hillocks and Thresh, 1998). This type is the most common in which lower leaves of the severely affected plants present a striking appearance in contrast to the fully green young leaves.

Stem symptoms

Stem symptoms are more apparent in the green tender portion of susceptible cultivars (Nichols, 1950a). Purple brown lesions are usually seen externally with deep penetration

reaching the cortex. Necrotic lesions may be vivid in the leaf scar after leaf abscission due to senescence (Hillocks and Thresh, 1998). In severe infections the necrotic lesions merge and expand to kill the axillary buds with subsequent shrinkage of nodes and death of internodal tissues (Hillocks, 1997). Ultimately, the branch dies from the tip downward, an effect described as ‘die back’ (Nichols, 1950a). Shoot symptoms are only critical when associated with die-back (Hillocks and Jennings, 2003). Symptoms are more apparent in cool dry weather than hot weather (Hillocks and Thresh, 1998).

Root symptoms

Necrosis in the storage root cortex is the most destructive of all symptoms that occur due to CBSD (Legg, 2003). Principally, the economic importance of the disease lies in the symptom expression in roots. The root symptoms usually develop after foliage symptoms (Hillocks and Thresh, 1998). The time taken from infection to the development of necrotic lesions in roots is cultivar-specific (Hillocks, 2003). Initially infected sensitive cultivars take five months from planting to CBSD root symptom expression whilst some tolerant cultivars take as long as eight months (Hillocks *et al.,* 1996).

CBSD-affected roots are often characterized by radial constriction accompanied with pits and fissures (Hillocks and Thresh, 1998). Brown to black stained tissues are usually observed surrounding the pitted portion (Nichols, 1950a). Internally, the yellow to brown, corky necrosis of starch-bearing tissues, characterizes the CBSD-affected roots. Lesions may be discrete but in susceptible varieties, whole portions of storage root become necrotic (Legg and Hillocks, 2003). In most cultivars, root constriction is not obvious but a cross-section through the root reveals

necrosis (Hillocks and Thresh, 1998; Nichols, 1950a). At an advanced stage, the necrotic lesions may attract pathologically important secondary organisms whose successful invasion leads to soft rot or decay of the root (Hillocks, 1997; Legg and Thresh, 2003).

Transient symptoms

Lack of consistent expression of symptoms in CBSV-infected plants is a common phenomenon (Jennings, 1960). In this category, symptoms may either be expressed for a short time during the plant growth or may not be expressed completely. According to Hillocks and Jennings, (2003), transient symptoms depend on three basic factors namely; i) cultivar response to infection or resistance, ii) prevailing temperature, and iii) crop growth stage.

CBSV-infected resistant cultivars tend to undergo active growth giving no time for the expression of CBSD symptoms (Storey and Doughty, 1951). The necrotic tissues are compressed against the bark and are frequently occluded (Hillocks and Jennings, 2003). This is common during re-growth triggered by rains after a period of reduced growth usually experienced in the dry season (Hillocks and Jennings, 2003; Legg and Hillocks, 2003).

Temperature is another important environmental factor that tends to trigger transient symptoms in CBSV-infected cassava (Nichols, 1950a). Severe expression of CBSD symptoms was reported at cool temperatures (Storey, 1936; Nichols, 1950a, 1950b). However, at high temperature, cassava plants grow more vigorously producing symptom-free leaves (Hillocks and Jennings, 2003; Storey, 1939). When high temperature coincides with new sprouting in plants whose older symptomatic leaves have been shed, the plant may appear CBSV-free.

Plant age also affects symptoms expression CBSD-affected plants (Hillocks *et al.,* 1999). The leaf symptoms becomes more difficult to recognize in older plants as most of the lower leaves are shed (Hillocks and Jennings, 2003). Under these circumstances, die-back remains the only viable option as an above ground diagnostic symptom (Nichols, 1950a). Since die-back is not a common character in most varieties (Hillocks, 1997), the observed plants may be regarded free from CBSV despite being infected.

Cultivar characteristics

Variation among cassava cultivars in expressing root and foliage symptoms of CBSD has been reported (Jennings, 1957, 1960; Storey and Doughty, 1951). The inherent characteristics of the susceptibility or resistance of cultivars leads to varied response to CBSV infection (Hillocks *et al.,* 2001). Most susceptible cultivars exhibits pronounced foliage and root symptoms, and the syndrome begins soon after sprouting in the cutting-derived infection. Some cultivars develop mild root symptoms without foliage symptoms (Hillocks and Jennings, 2003). Apparent foliage symptoms without or with delayed root symptoms may be seen in a few cultivars like Nachinyaya (Hillocks *et al.,* 2001).

Altitude

Almost all published reports about CBSD have indicated the influence of altitude on the incidence and severity of the disease (Hillocks, 1997; Jennings, 1957; Legg and Raya, 1998; Nichols, 1950a). CBSD was originally common at altitude below 1000 masl, at which temperature conditions are considered ideal for the spread of the virus (Nichols, 1950a).

However, symptoms of the disease have since been observed at higher altitudes in Uganda and Tanzania (Alicai *et al.*, 2007; Legg and Raya, 1998; Nichols, 1946).

Jennings (1960) reported the presence of CBSD at 1630 masl, with root necrosis more apparent than shoot symptoms. However, limited spread of the disease was recorded. The effect of altitude on CBSD could be indirect, through temperature variation that affects both vectors and replication of the virus (Nichols, 1946).

Environmental conditions

The environmental parameter reported to have the greatest influence on CBSD symptoms expression is temperature (Hillocks and Jennings, 2003; Nichols, 1950a). Moisture stress was also suspected to influence the severity of CBSD in affected plants (Nichols, 1950b). Low temperature augments the effects of the disease to the extent that highly intolerant clones may even be killed after infection (Nichols, 1946; Storey, 1939). Low temperature is assumed to trigger fast response to the disease, but high temperature delays symptom development. Despite this general knowledge over the influence of temperature, the critical ranges at which temperature trigger changes in symptoms development are not known.

Suspicion of the possible influence of moisture stress on CBSD symptoms was indicated by Storey (1939). Severe symptoms were recorded in experiments conducted in Tanga for three consecutive years whereby the common factor at all sites was drought (Nichols, 1950b). CBSD-affected plants manifested severe conspicuous root necrosis such that their value as food was nil. Although temperature was relatively high even in cool seasons, a prolonged dry spell led to

increased root damage of affected plants. The suspected influence of drought on severity of CBSD has never been experimentally justified.

1. 2. 11 Economic importance of CBSD

Leaf necrosis and stem lesions expressed by infected susceptible cultivars (Nichols, 1950a) are considered important because they may impair the photosynthetic efficiency of the plant. This is particularly true in sensitive cultivars in which numerous necrotic lesions develop in young leaves. However, root necrosis is the most damaging symptom (Hillocks *et al.*, 1999, 2001; Hillocks and Thresh, 2003; Jennings, 1957; Nichols, 1950a). Susceptible cultivars have been reported to sustain root yield loss of up to 70 % per plant (Hillocks *et al.,* 2001). The disease has transformed the long-term chronic food shortage in Malawi (Shaba *et al*., 2003) and Mozambique (Steel, 2003) into an acute one. In Uganda, the success in overcoming the CMD-UgV pandemic that ravaged the country in 1990s has been short-lived, because many of the CMD-resistant cultivars that have been deployed have been reported to be susceptible to CBSD (EARRNET, 2005; Alicai *et al.,* 2007). CBSD attack has devastated cassava growers due to the fact that cassava is a food security and income generation crop in the affected countries (Legg and Hillocks, 2003). Hunger and the lost household income have left many families in total dismay (Pearce, 2007).

1. 2. 12 Diagnosis and detection of *Cassava brown streak virus*

CBSD is a poorly studied disease despite being known for more than seventy years (Maruthi *et al.,* 2005). Limited progress has been made in diagnosis and detection of the disease. Much of the published work on CBSD diagnosis concentrated on observable symptoms on the

shoot and root parts. The first scientific guide on detection of the disease was developed in 2002 (Legg and Hillocks, 2003). This guide still capitalizes on symptomatology descriptions and some molecular techniques. Unfortunately, dependency on symptom expression fails to detect latently infected plants unless robust molecular techniques are also used. Unfortunately, these techniques are expensive and high-tech-based.

Field diagnosis

Reported studies on symptoms indicate that CBSD is expressed in leaves, stem and roots of susceptible cultivars (Nichols, 1950a). This knowledge of disease symptoms created a dependable basis for field diagnosis of the disease. Unfortunately, it has also been observed that CBSD symptoms are complex and difficult to diagnose (Jennings, 1957). Some cultivars manifest symptoms similar to the Nichols (1950a) description of CBSD but may test negative to the virus (G. Rwegasira, unpublished data). Apart from the complexity of symptoms associated with the disease, CBSV does not affect cassava in isolation from other factors. A combination of both abiotic stress (nutrients imbalance, salinity, draught and soil moisture) and biotic stress such as bacteria, fungal, physiological ageing, sucking insects (mites, scale, mealy bugs) might collectively affect the plant to cause mixed foliage symptoms. The resultant combination of symptoms may obscure important observations and lead the observer to incorrect diagnosis.

In addition, not all CBSV-infected plants develop CBSD symptoms. Some plants exhibit latency as their inherent genetic response in the prevailing environmental condition (Hillocks and Jennings, 2003). The symptom complications in which CBSV-infected plants remain symptomless makes it difficult to truly conclude on the health status of the plant. It becomes

necessary that symptom-based diagnosis is supplemented with robust CBSV-detection techniques. Unfortunately, the best sample to collect for laboratory detection of the virus has never been identified.

Laboratory detection of CBSV by RT-PCR

The molecular technique for CBSV detection by reverse transcriptase polymerase chain reaction (RT-PCR) was developed by Monger *et al.,* (2001a). Using Coat Protein gene-specific primers for CBSV, the virus was isolated from infected samples and sequenced. The development improved precision in the diagnosis of the virus. Using this technique, two CBSV strains were delineated (Monger *et al.,* 2001b). The robustness and precision of the molecular technique has promoted its wide use. In Uganda, the technique was used effectively to detect and confirm the presence of CBSV in infected plants (Alicai *et al.*, 2007). Despite the robustness, the technique is highly advanced and costly, which limits its use for regular detection of the virus.

1. 2. 13 Human interventions

The intervention of farmers and researchers to mitigate yield loss sustained from CBSD started soon after the disease was reported (Storey, 1936; 1939). Massive importation of cassava clones from wherever cassava is grown and screening for resistance was done (Nichols, 1947). Detailed study of the disease involved repeated experimentation, to understand the disease and develop possible management techniques. Breeding for resistance (Jennings, 1957; Storey and Doughty, 1951; Storey, 1936), phytosanitation (Nichols, 1957) and quarantine (Nichols, 1950a) were the major undertakings in attempts to manage CBSD.

Breeding for resistance

Efforts to manage CBSD through resistant cultivars dates back to the 1930s, when a world-wide collection of accessions of cassava was evaluated in attempts to identify sources of resistance (Jennings, 1957; Nichols, 1947). Resistance was later identified in *Aipin valenca* (obtained from Belgian Congo) and *Macaxeira aipin* (from Brazil) (Nichols, 1947). Using these varieties, many resistant cultivars were developed by conventional breeding and maintained at Amani, hence popularly known by breeders as the Amani hybrids (Mahungu *et al.*, 1999). Although very useful, the hybrid varieties were not formally maintained probably due to the fact that CBSD had not become a serious field problem. This complicated the breeders' work as they started looking for resistance in farmer-grown cassava clones. In the late 1980s when CBSD was reported as a threat in Mtwara region (Southern Tanzania), breeders evaluated varieties collected from farmers' field for field resistance to CBSD. This allowed them to identify few promising cultivars including Kigoma red, Nanchinyaya and Namikonga (Kanju *et al.*, 2003). Most of these cultivars exhibited partial resistance and are believed to have originated from Amani.

Recovery from disease or field resistance refers to the phenomenon whereby the previously diseased plants tend to recover from the infection when planted in a new field environment and there is arrested manifestation of the disease symptoms (Fargette *et al*., 1996; Jennings, 1957). It is often signified by visible necrotic local lesions and no virus-related symptoms are observed. The failure by the host plant to recognise the virus infection thereby not allowing any replication or systemic movement in the cells, even in the initially inoculated cells, signifies immunity (Agrios, 2005; Matthews, 2002).

Phytosanitation

Apart from breeding, selection and roguing have been attempted with less success because different cultivars vary in symptom expression (Hillocks *et al.*, 1999). The relationship between cultivar characteristics and seasonality in expression of CBSD symptoms is not well understood. Most farmers tend to select planting materials at harvesting when most of CBSD symptomatic leaves have been shed (Katinila *et al*., 2003). This makes it difficult to avoid using the infected materials. In many instances, farmers tend to borrow propagation materials from each other, increasing the opportunity for movement of infected cuttings. In some areas planting materials are usually scarce and inadequate at planting time (Kanju *et al*., 2003; Katinila *et al*., 2003). This situation forces farmers to unknowingly collect CBSD-affected cuttings.

In cultivars which manifest CBSD foliage symptoms, the collection of healthy stems may be supplemented with roguing of symptomatic plants at sprouting. The efficiency of this technique depends on the sensitivity of the grown cultivar and the apparency of the CBSD symptoms (Hillocks and Thresh, 1998). The prevailing temperature may also be a vital determinant of phytosanitation efficiency, given its suggested influence (Nichols, 1950a) on CBSD symptoms expression.

Plant quarantine

Plant quarantine refers to the legally enacted measures to restrict or prohibit movement of agricultural products (live or dead) to avoid spread of noxious pests and diseases into new locations that are free from infection (FAO, 1995). It is a first line of defence, issued legislatively and intended to exclude pests from the host or geographic area (Ebbels, 2002). Legislation

establishes the statutory authority for the government to engage in limiting further disposal of the pest or treating the localized infestation (Mohamed, 2003). The documented evidence of quarantine, imposed to restrict further spread of CBSD was in Uganda in 1948/49, after the disease was suspected to have been introduced through infected breeding material planted at Bukalasa Research Station (Nichols, 1950a). In the most recent phase of the studies of CBSD, assistance was sought from a plant health centre (KEPHIS) at East Africa Headquarters in Kenya to assist in indexing and screening of planting materials intended to be moved or exchanged within the region (Onamu *et al.*, 2003). This will ensure that exchange of cassava germplasm in East Africa is through tissue culture, accompanied with phytosanitary certificate from authorities in the respective countries.

1. 3 Aims of the study

The current studies aim at establishing facts on CBSD epidemiology and create a fundamental basis for developing appropriate management strategies of the disease.

Specific aims

1. To determine the current status of CBSD and the diversity of CBSV in Tanzania.
2. To identify the non-vector mechanisms of CBSV transmission and determine their features and efficiency.
3. To explore the diversity of CBSD symptoms and establish the relationship between symptoms and CBSV infection.
4. To identify the factors that affect disease severity in CBSD-affected plants.

CHAPTER 2

THE STATUS OF CASSAVA BROWN STREAK DISEASE AND DIVERSITY OF *CASSAVA BROWN STREAK VIRUS* (*POTYVIRIDAE: IPOMOVIRUS*) IN TANZANIA

Manuscript submitted to Phytopathology Journal

2. 1 SUMMARY

Cassava brown streak disease (CBSD) in Tanzania was known for many years to cause damage only in low altitude coastal areas (less than 500 metres above sea level). A survey was conducted in 2005 in the Coastal, Lake and Southern Zones, to assess the status and geographical distribution of the disease in the major cassava growing areas of Tanzania. The survey was repeated in a few sites in the Lake and Southern Zones in 2006. A total of 2730 cassava plants were assessed for CBSD leaf symptoms in 91 fields with an average of 30 plants per field. At each site, a sample was taken for laboratory testing for *Cassava brown streak virus* (CBSV). CBSD root symptoms were assessed in plants at eighty one sites. CBSD mean foliage and root incidences were 38 % and 36 %, respectively. CBSV tests by replicated RT-PCR, using CP-specific primers, indicated the presence of CBSV in 67 of the 91 (73 %) samples of which 61 samples were expressing symptoms. Forty three amplicons were sequenced and the phylogenetic analyses suggested that one major clade of CBSV exists in Tanzania. Forty two of 43 sequences clustered together with six CBSV sequences (from Mozambique and Tanzania) in a public database with 80-100 % nucleotide identity. Thirteen of 42 isolates had less than 80 % nucleotide identities with the three Ugandan CBSV sequences in the public database. One isolate, FJ687177 differed from the rest of Tanzanian sequences with 77.4 % nucleotides identity but closely

related (93.4 %) to the Ugandan isolates in the public database. CBSD was widely distributed across the country including sites approximately 1800 metres above sea level (masl). CBSD is reported for the first time in the Lake Zone of Tanzania.

2. 2 INTRODUCTION

CBSD was first reported in coastal Tanganyika (now Tanzania) more than 70 years ago (Storey, 1936). The disease was common at altitudes below 1000 masl, and serious damage occurred at elevations below 500 masl (Nichols, 1950a; Storey, 1939). The geographical distribution of CBSD included all of the East African coastal areas extending from the north-east border of Kenya to Mozambique and the low altitude areas in Nyasaland (now Malawi) particularly towards the southern border with Mozambique (Nichols, 1950a).

Early studies on the possible causal agent, mechanism of spread, and management of the disease through resistant cultivars were conducted at Amani Research Station, in north-eastern colonial Tanganyika (Jennings, 1957; Nichols, 1950a; Storey, 1936). The first detailed description of the disease was given (Nichols, 1950a). The brown mass of necrotic inclusions in the root cortex of affected plants was established as the most damaging symptom associated with the disease (Storey, 1936).

Widespread occurrence of CBSD was reported in later surveys (Legg and Raya, 1998; Legg and Hillocks, 2003), but mainly restricted to the eastern coastal areas of Tanzania including Zanzibar Islands. The disease was reported to be disseminated in infected cuttings including the

earlier accidental introduction of the CBSD in Uganda through breeding materials sent to Bukalasa station from Amani (Nichols, 1950a; Storey, 1939). Several vectors such as whitefly, *B. tabaci* and *B. afer* (Storey, 1939; Bock, 1994) and aphid, *Myzus persicae* Sulz. (Lennon *et al*., 1986) were also suspected to aid the spread of CBSV. The whitefly vector, *Bemisia tabaci* Gennadius (*Hemiptera: Aleyrodidae*) was later implicated in CBSV spread albeit at low mean transmission efficiency *ca* 2 % (Maruthi *et al.*, 2005). Recent reports on CBSD in Uganda (Alicai *et al.*, 2007; EARRNET, 2005) confirmed that the disease has spread into areas of about 1200 masl. This suggested a significant change in CBSV strains and the environment that favours CBSD development.

In the current study, the status of CBSD and CBSV strain diversity in Tanzania were determined. The specific objectives were:

(i) To determine the incidence and severity of CBSD in farmers' cassava fields in the main cassava growing areas of Tanzania,

(ii) To establish the current geographical distribution of CBSD in Tanzania,

(iii) To determine the diversity of CBSV strains in Tanzania.

2. 3 MATERIALS AND METHODS

2. 3. 1 Field survey

A total of 2,730 plants in 91 farmers' cassava fields in nineteen regions were assessed for incidence and severity of CBSD in a survey conducted in all major cassava growing areas between August and October 2005. The survey was repeated at a few sites of the Lake and

Southern Zones in June, 2006. Three major Zones for cassava growing designated as 'Coastal' (coastline stretch along Indian Ocean extending from north-east in Tanga region to southern-east in Mtwara), 'Lake' (along the Lake Victoria basin in the north-western part extending to the northern shore of Lake Tanganyika), and the 'Southern' zones (south to western stretch from the Mozambique border through the shore of Lake Malawi to the southern shore of Lake Tanganyika) were covered. Surveyed Regions included: Coast, Morogoro, Lindi, Mtwara, Tanga, Zanzibar and Mafia archipelagos (Coastal Zone), Kagera, Kigoma, Mara and Mwanza (Lake Zone), and Iringa, Mbeya, Rukwa and Ruvuma (Southern Zone). Cassava fields were surveyed along accessible roads by stopping at regular intervals of *ca*100 kilometres. Only fields with crops older than three months were assessed and sampled. A total of 30 plants were assessed for CBSD foliage and stem symptoms, along the diagonal of each field. Based on farmers' names (some synonyms) of the cassava being grown, 64 different cultivars were assessed.

2. 3. 2 Field assessment of CBSD

Field incidence and severity of CBSD were recorded based on shoot, stem and root symptoms. Incidence was considered as the percentage of the total number of plants with CBSD symptoms. A Natural Resources Institute (NRI) scale of 1 (no symptoms) through 5 (severe symptoms) (Hillocks and Thresh, 1998), was used with minor modifications to assess severity. The key to CBSD incidence and severity scores used in this study is presented (Table 2.1).

Assessments for root symptoms were done in 81 of the 91 fields. One to fifteen (average of five) plants were uprooted in each field and assessed. All the roots on each uprooted stem

were counted, three to several cross section cuts were made, near the top, middle and tip of each tuberous root, and the proportion of necrotic ones was used to determine incidence. The proportion of tissues damaged by necrosis was used as the root severity score. The shoot and root incidence and severity of CBSD on the uprooted plants were also recorded.

In plants that were dually-affected by CBSD and CMD, the incidence and severity of the CMD were also assessed. The number of adult whitefly, *B. tabaci,* was counted and recorded from the top five fully-expanded leaves of each of the thirty plants and averaged. Whenever a mixture of varieties was found in the same field, the most common one was assessed. The location of each sampling site was recorded using a global positioning system (GPS) (Garmin®, 2006) and used to generate maps with ArcGIS 9.2 (ESRI, 2006).

2. 3. 3 Sample collection and handling

Leaf samples collected during the field survey were placed into self-sealable plastic bags and the air squeezed out. One leaf sample was collected from a single plant in each field. Leaves with CBSD symptoms were sampled preferentially. In the absence of symptomatic leaves any plant was selected. The fourth or fifth fully-open leaf from the top was sampled. Collected samples were stored under ice in a cool box and sent to Mikocheni Agricultural Research Institute in weekly batches where they were ground in liquid nitrogen and stored at –80 ^{0}C.

Table 2.1 Description of visual diagnostic scale for CBSD used during the field survey

Disease parameter	Plant part assessed	symptomatic /damaged	Score scale	Description
CBSD incidence	Foliage	0 %	1	None of the leaves has chlorosis characteristic for CBSD
		1 - 5 %	2	Very few leaves show faint chlorosis/blotches
		5 - 30 %	3	Appreciable number of leaves show chlorosis/blotches
		30 - 50 %	4	Half the leaves show chlorosis/blotches
		50 – 100 %	5	Almost all leaves show CBSD chlorosis
	Stem	0 %	1	No necrotic spot or lesion characteristic for CBSD seen on stem
		1 - 5 %	2	A small part of the stem/one branch has necrotic spot
		5 - 30 %	3	Appreciable part of the stem has necrotic spot(s)
		30 - 50 %	4	Necrotic spot(s)/lesions apparent on stem(s)
		50 – 100 %	5	Necrotic spot(s)/lesions apparent of all branches/stems
	Root	0 %	1	None of the roots has necrosis characteristic for CBSD
		0 - 5 %	2	Very small number of roots have necrosis characteristic for CBSD
		5 - 30 %	3	Appreciable roots have necrosis characteristic for CBSD
		30 - 50 %	4	Most roots have necrosis characteristic for CBSD
		50 – 100 %	5	Almost/all roots have necrosis characteristic for CBSD
CBSD severity	Foliage	0 %	1	None of the leaves has chlorosis characteristic for CBSD
		1 - 5 %	2	Slight chlorotic spots characteristic of CBSD seen on leaves
		5 - 12 %	3	CBSD chlorotic spots/blotches easily observable on leaves
		12 - 30 %	4	Appreciable CBSD chlorotic spots/blotches seen on leaves
		30 – 100 %	5	Very severe chlorotic/necrotic blotches and leaf wilt
	Stem	0 %	1	No necrotic spot or lesion is seen on stem
		1 - 5 %	2	Slight chlorotic spots on tender portion of the stem
		5 - 12 %	3	Necrotic spots are numerous, coalesced to small lesions
		12 - 30 %	4	Severe necrotic lesions enlarged into streaks
		30 – 100 %	5	Severe necrotic lesions, streaks, withering and die-back
	Root	0 %	1	None of the roots has necrosis characteristic for CBSD
		1 - 5 %	2	Small portion of roots bears necrotic spots
		5 - 12 %	3	Appreciable proportion of the roots is obviously necrotic
		12 - 30 %	4	Roots mostly necrotic, not suitable for consumption
		30 – 100 %	5	Roots are almost/totally necrotic, started rotting

2. 3. 4 RNA extraction and RT-PCR

RNA was extracted from approximately 0.1g of tissue from each of the samples using Pure Link^{TM} Plant RNA purification reagent as per manufacturer's instructions (Invitrogen Life Science, Cat. 12322-012). The extracted RNA pellet was dissolved in 35 µl of pure RNase-free water and kept at –80 ^{0}C as stock for RT-PCR amplification. Samples from plants known to be CBSV-positive were included as positive controls. In addition, samples from cassava seedlings grown in a screen-house were used as negative checks.

2. 3. 5 cDNA synthesis and amplification by RT-PCR

Triplicate reverse transcriptase polymerise chain reaction (RT-PCR), was used in a one-step reaction using superscript ^{TM} III RT/Platinum® *Taq* Mix System (Invitrogen Life Technologies, Cat. PN.52122) using a GeneAmp PCR system 9700 (Applied Biosystems, UK). The CBSV coat protein (CP)-specific primers, CBSV 10/F (5'-ATCAGAATAGTGTGACTGCTGG-3') and CBSV 11/R (5'-CCACATTATTATCGTCACCACG-3') (Monger *et al.*, 2001a) were used. The primers were designed to amplify an approximately 231 bp of the most conserved CP gene fragment. PCR conditions were as follows: initial cDNA synthesis and denaturation at 55°C for 30 minutes and 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min (in 35 cycles) and final extension at 72°C for 10 min.

2. 3. 6 RT-PCR product analysis

RT-PCR products were separated electrophoretically in 1.2 % agarose gels in 0.5X Tris borate EDTA (TBE) buffer, for 1 hour at 92 volts. The products were visualised by staining with

ethidium bromide (0.01 µl/ml) under ultraviolet (UV) light and recorded using an image analyser.

2. 3. 7 Sequencing of amplicons

A total of 43 amplicons were directly sequenced at the International Livestock Research Institute (ILRI) laboratory in Nairobi, Kenya. The ABI-generated files were aligned and edited using Sequencher software version 4.6 (Gene Codes Corporation, USA). The sequences were aligned using Clustal X 1.83 (Sci-biology, 2005) and further editing done in Bioedit (Hall, 1999). Each sequence was subjected to BLAST searches (Zhang *et al*., 2000) in the National Centre for Biotechnology Information (NCBI) database. The nine CBSV coat protein sequences published in the NCBI were retrieved and aligned together with the 43 newly obtained sequences (Sci-biology, 2005). Available sequences were; AF311052, AF311053 (Mozambique), AY007597, AY008440, AY008441, AY008442 (Tanzania) all by Monger *et al.* (2001), and DQ837302, DQ837303 and DQ837304 (Uganda) by Alicai *et al.*, (2007). CP sequences for one Kenyan CBSV isolate (KECBSV) were kindly provided by Dr. Munga T, of KARI-Mtwapa. Comparable sequences of the two related ipomoviruses; *Cucumber vein yellowing virus* (CVYV) (DQ287320) and *Sweet potato mild mottle virus* (SPMMV) (Z48058) and one Potyvirus; *Tobacco etch virus* (TEV) (DQ871334) were also obtained and aligned together with the CBSV sequences for comparisons (Table 1; appendix 1). The phylogenetic analysis was done through Neighbour Joining methods (NJ) with 1000 bootstrap replications in PAUP* 4.0 Beta (Swafford, 2003) and re-checked using a maximum likelihood method. The resultant phylogram were visualised in Tree View 1.6.6 (Hall, 2004). The multiple comparisons of sequences were

performed in MegAlign (DNA* Star) and the percentage similarities among nucleotides and amino acids estimated.

2. 3. 8 Data analysis

The survey data were analysed using GenStat 4.24DE (GenStat, 2005) and the Statistical Package for Social Sciences (SPSS 12.0) (SPSS, 2003). Pearson's correlation '*r*' analysis was done on CBSD, CMD and *B. tabaci* parameters to determine the significantly related variables (goodness of fit, $P < 0.05$).

2. 4 RESULTS

2. 4. 1 Geographical distribution of CBSV

A map was constructed based on the GPS coordinates of sample points and RT-PCR test results and it indicates the presence of CBSV in many parts of the country (Figure 2.1). Most of the collected samples, 67 of 91 (73.6 %) samples tested positive for CBSV suggesting that, it occurs in all the major cassava-growing areas around the country. A total of 81 of 91 samples had foliage symptoms, although CBSV was detected in only 61 samples accounting for 67 % of apparent infection. CBSV was also detected in six of ten samples without foliage symptoms. Only a few, 24 out of 91 (26.4 %) samples were free from the virus. Based on CBSV-infection data, the infection rates did not vary considerably among the three zones. CBSV was detected in 22 of 28 (78.6 %) samples from the Eastern Zone, 26 of 36 (72.2 %) samples from the Lake Zone and 19 of 27 (70.4 %) samples from the Southern Zone. This contributed to the country mean infection rate of 73.6 %.

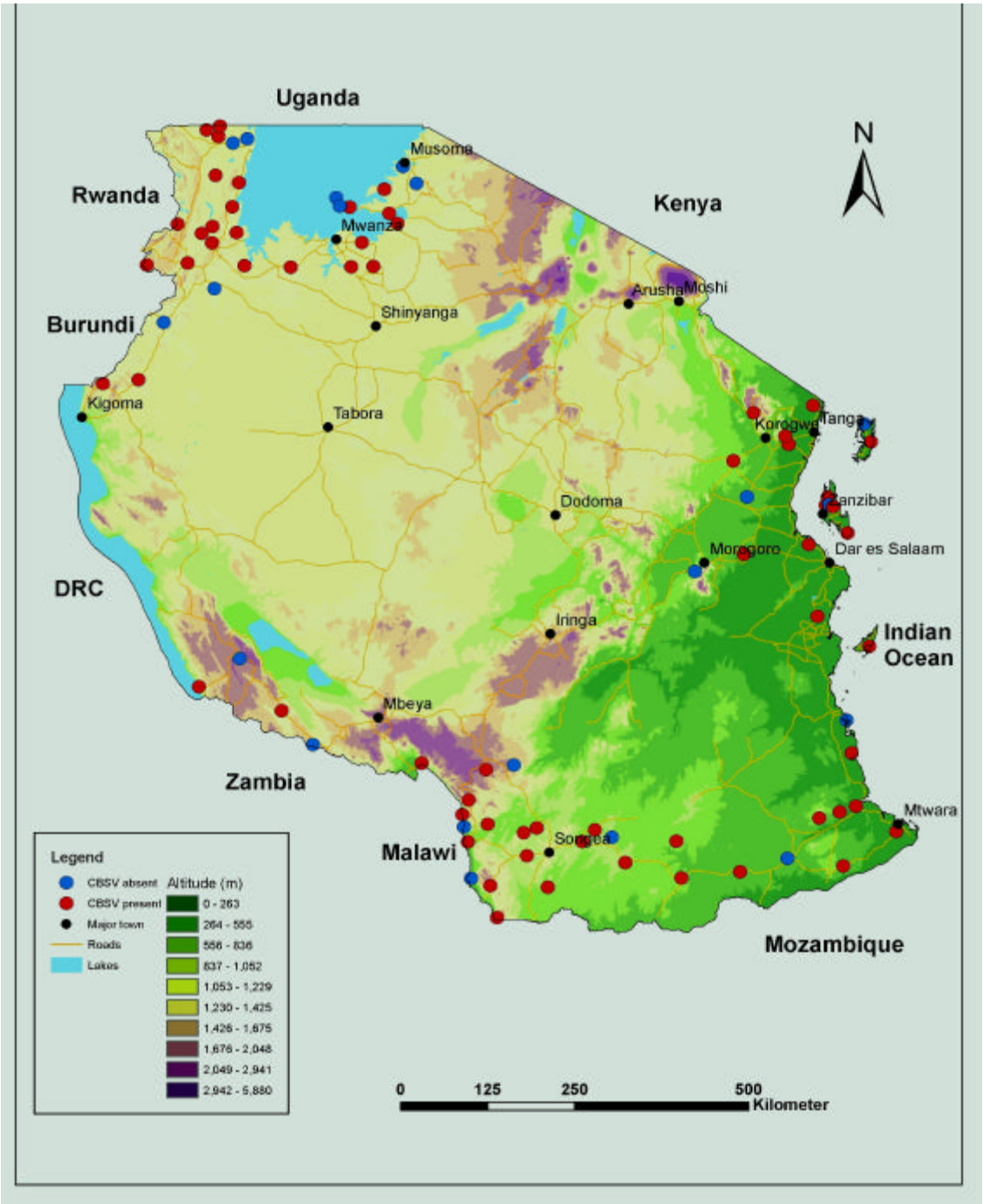


Figure 2.1 Distribution of *Cassava brown streak virus* in Tanzania as detected by RT-PCR. One plant was sampled from each site and the virus detected from 0.1 g of finely-ground leaf sample by RT-PCR using CBSV CP-specific primers.

2. 4. 2 Confirmation of CBSV in collected samples

An expected amplicon consisting of *ca* 230 bp was obtained in RT-PCR (Figure 2.2). The band strength for the tested samples was not uniform although the same method of RNA isolation was used throughout. Sample 5L obtained from a symptomless plant (at Chato, in the Lake Zone), had a brighter band than the rest of samples, including the positive control.

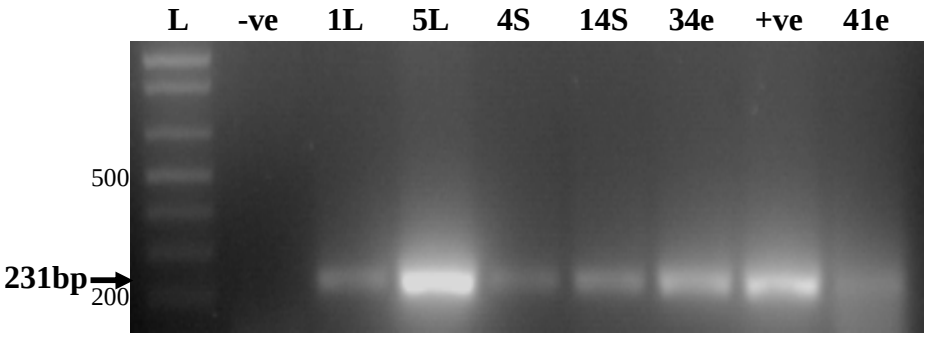


Figure 2.2 Agarose gel electrophoresis of RT-PCR amplified products (231bp CBSV coat protein gene fragment) for representative samples using CBSV-specific primer pair CBSV 10F and CBSV 11R. RNA was isolated from 0.1 g of leaf tissue from collected samples; "L", 1kb plus DNA marker (Invitrogen, cat.10787-018); "–ve", negative control (tissue culture), "1L and 5L" Sample from Ukerewe Island (cv. Rwabukindo) and Chato (cv. Mdalla); "4S and 14S" samples from Mbinga (cv. Eliza) and Sumbawanga (cv. Kalulu); "34e and 41e" samples from Tanga (cv. Mahiza) and Zanzibar (cv. Mtaimbo); and "+ve", positive control from CBSV-infected cultivar from SRI-Kibaha (cv. Albert).

2. 4. 3 Phylogenetic analysis of CBSV amplicons

The phylogenetic analysis of coat protein gene amplicons from Tanzania (Figure 2.3) suggested that CBSV sequences have limited diversity. One of 43 (2.3 %) CBSV isolates, (FJ687177), from Tanzania cladded differently from the rest of the isolates. This isolate was

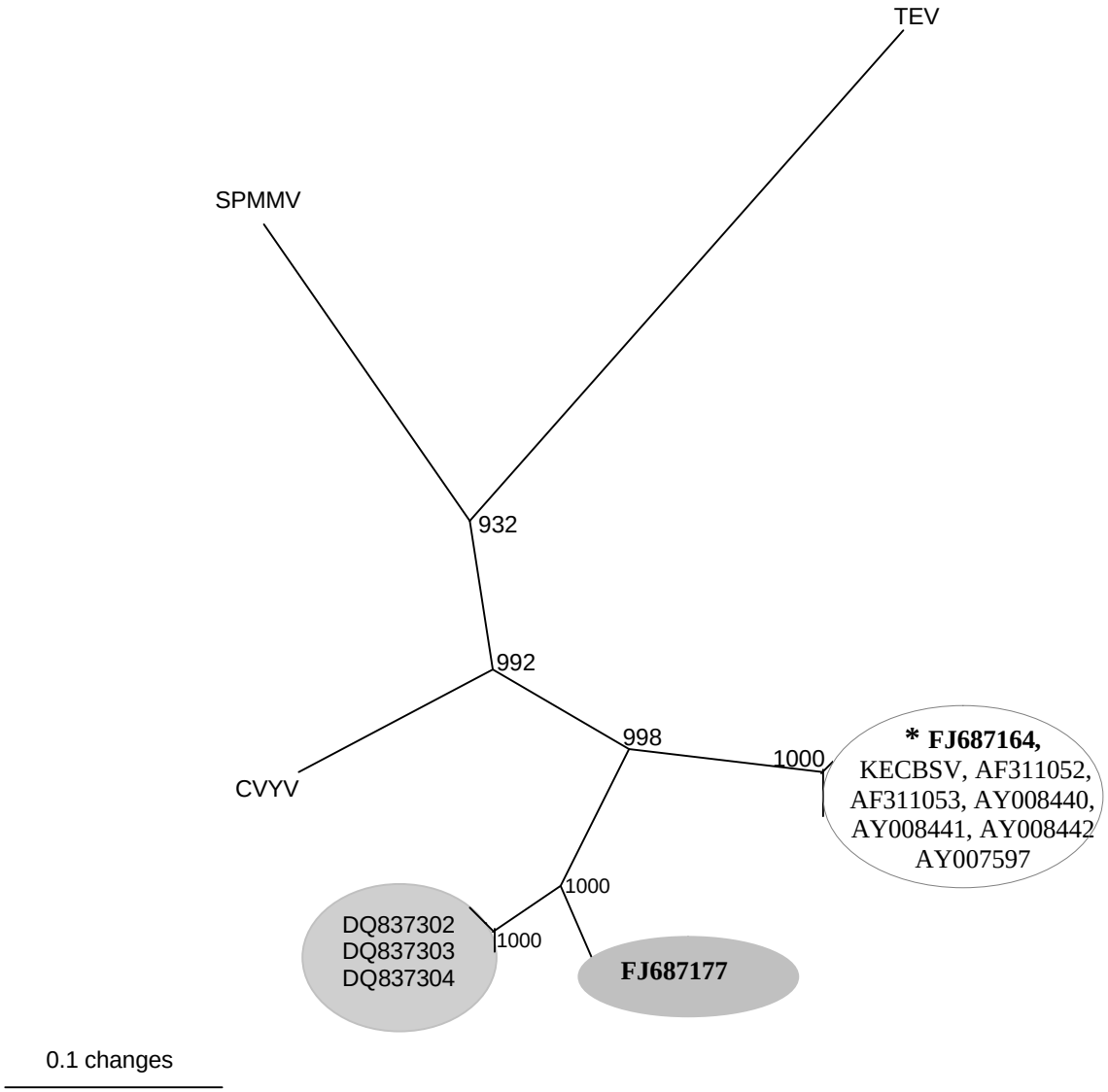
obtained from a local variety in Masasi, in the southern part of Tanzania, and was closely related to the Ugandan isolates, DQ837302, DQ837303 and DQ837304. Surprisingly, the location from which this isolate was collected is geographically remote from Uganda.

Table 2.2 List of the 43 CBSV isolates collected during the survey in Tanzania, the cultivars and status of CBSD symptoms on the sampled plants

Sequence name	Source cultivar	District (Location)	Foliage symptoms	Root symptoms	Country/ source
FJ687164	Albert	SRI-Kibaha	+	+	Tanzania
FJ687165	Albert	Bagamoyo	+	+	…..,,…..,,.
FJ687166	Agriculture	Bagamoyo	+	-	…..,,…..,,..
FJ687167	Kiba'meno	Muheza	+	+	…..,,…..,,.
FJ687168	Msese	Handeni	+	+	…..,,…..,,.
FJ687169	M'kuonja	Bagamoyo	+	+	…..,,…..,,.
FJ687170	Kipusa	Magharibi	+	+	…..,,…..,,.
FJ687171	Mwari	Chakechake	+	+	…..,,…..,,.
FJ687172	Boma	Kaskazini B	+	+	…..,,…..,,.
FJ687173	Kiroba	Mkuranga	+	+	…..,,…..,,.
FJ687174	Mbuyu	Kilwa	+	+	…..,,…..,,.
FJ687175	Ayuda	Lindi	+	+	…..,,…..,,.
FJ687176	Nanchinyaya	Mtwara	+	+	…..,,…..,,.
FJ687177	Mreteta	Masasi	+	+	…..,,…..,,.
FJ687178	Mzungu	Mkuranga	+	+	…..,,…..,,.
FJ687179	Rwabukindo	Ukerewe	+	+	…..,,…..,,.
FJ687180	Toboka	Geita	+	+	…..,,…..,,.
FJ687181	Mdalla	Chato	-	-	…..,,…..,,.
FJ687206	Bukalasa	Bukoba	+	+	…..,,…..,,.
FJ687182	Bukalasa	Bukoba	+	+	…..,,…..,,.
FJ687183	Unknown	Misenyi	+	-	…..,,…..,,.
FJ687184	Unknown	Misenyi	+	+	…..,,…..,,.
FJ687185	Unknown	Muleba	+	+	…..,,…..,,.
FJ687186	Kataakya	Muleba	+	+	…..,,…..,,.

FJ687187	Lumala	Muleba	+	-	…..,,…,,.
FJ687188	TMS 4(2) 1425	Biharamulo	+	+	…..,,…,,.
FJ687189	TMS 4(2) 1425	Ngara	+	+	…..,,…,,.
FJ687190	Ndelela	Kasulu	+	-	…..,,…,,.
FJ687191	Kongo	Kigoma	+	-	…..,,…,,.
FJ687192	Muganda	Misungwi	+	+	…..,,…,,.
FJ687193	Lyongo	Magu	+	-	…..,,…,,.
FJ687194	Lwabakanga	Bunda	-	-	…..,,…,,.
FJ687195	Kachaga	Musoma	+	-	…..,,…,,.
FJ687196	Mipeta	Mbinga	+	-	…..,,…,,.
FJ687197	Mtutuma	Ludewa	+	+	…..,,…,,.
FJ687198	Paliani	Mbinga	+	-	…..,,…,,.
FJ687199	Mwaya	Namtumbo	+	+	…..,,…,,.
FJ687200	Kigoma	Tandahimba	+	-	…..,,…,,.
FJ687201	Mwaya	Ruvuma	+	-	…..,,…,,.
FJ687202	Dide red	Tunduru	+	-	…..,,…,,.
FJ687203	Kayumu	Songea	+	+	…..,,…,,.
FJ687204	Kifuu	Ludewa	+	+	…..,,…,,.
FJ687205	Kabumba	Sum'wanga	+	-	…..,,…,,.
	REFERENCE ISOLATES				
±KECBSV	Guzo	N/A	+	N/A	Kenya
*AF311052	Mulaleia	Zambezia	+	N/A	Mozambique
*AF311053	Fernando	Zambezia	+	N/A	Mozambique
*AY007597	Unknown	Unknown	N/A	N/A	Unknown
*AY008440	Mukukumkuk	Unknown	+	N/A	Tanzania
*AY008441	Vumbi	Unknown	+	N/A	Tanzania
*AY008442	Kibaha	SRI-Kibaha	+	N/A	Tanzania
*DQ837302	TMS I 92/0057	Mukono	+	N/A	Uganda
*DQ837303	Ebwan'tereka	Namulonge	+	N/A	Uganda
*DQ837304	TME 204	Wakiso	+	N/A	Uganda

A total of 43 out of 67 CBSV-positive samples were sequenced. CBSD symptoms on the 43 plants from which the samples were obtained were as follows: 28 plants had both foliage and root symptoms, 13 had only foliage symptoms, 2 plants were symptomless and none of the plants had root symptoms without foliage symptoms. The "+" sign indicate presence; "-" indicate absence; N/A indicate not applicable; "±" CBSV CP gene sequences for an isolate from Kenya and "*"already published CBSV-CP gene sequences obtained from NCBI public database.



*Represents other isolates of the same clade which includes:
FJ687165, FJ687166, FJ687167, FJ687168, FJ687169, FJ687170, FJ687171, FJ687172, FJ687173, FJ687 174, FJ687175, FJ687176, FJ687177, FJ687178, FJ687180, FJ687181, FJ687182, FJ687183, FJ687184, FJ687185, FJ687186, FJ687187, FJ687188, FJ687189, FJ687190, FJ687191, FJ687192, FJ687193, FJ687194, FJ687195, FJ687197, FJ687198, FJ687199, FJ687200, FJ687201, FJ687202, FJ687203, FJ687204, FJ687205 and FJ687206.

Figure 2.3 Bootstrapped phylogenetic tree of partial coat protein gene nucleotide sequences encoding nucleotide sequences of 46 Tanzanian and seven other isolates of *Cassava brown streak virus*. Sequences for the two ipomoviruses (*Cucumber vein yellowing virus* and

Sweetpotato mild mottle virus) and one potyvirus (*Tobacco etch virus*) were included as outliers. Only bootstrap values exceeding 900 are shown.

Most isolates (97.7 %) formed a clade with the six published CBSV isolates from Mozambique and Tanzania (Genbank accession) in the NCBI database (Figure E1; appendix ii). Isolates in this clade were obtained from different cultivars grown in diverse locations throughout Tanzania (Table 2.2), in the Coastal Zone, Lake Zone and Southern Zones. One isolate from Kenya (KECBSV) was similar to the new Tanzanian isolates. The phylogenetic relationship showed intra-specific variation among sequences of the same clade, suggesting some diversity among the CBSV isolates.

Comparisons of the coat protein gene nucleotides (Table E2, Appendix iii) and amino acid sequence (Table E3, Appendix iv) identities among the new and published CBSV isolates showed that most isolates were diverse in nucleotide sequences but highly related in terms of amino acid identities. The nucleotide identities among the isolates in this study and the six published isolates (AF311052, AF311053, AY007597, AY008440, AY008441 and AY008442) from Mozambique and Tanzania all exceeded 80 % except one isolate FJ687177. In contrast, the nucleotide sequence comparisons between the new isolates and the three published Ugandan isolates (DQ837302, DQ837303 and DQ837304) suggested low identities. The new isolates: FJ687180, FJ687182, FJ687183, FJ687187, FJ687188, FJ687189, FJ687190, FJ687194, FJ687198, FJ687199, FJ687200, FJ687204, FJ687205, FJ687206 and KECBSV had nucleotide identities less than 80 % to the Ugandan isolates.

The amino acids sequence comparison suggested that the majority of new CBSV isolates were highly identical (> 80 %) to all the published isolates. Interestingly, most of the new CBSV isolates had close amino acids identity (> 80 %) to CVYV, except the four isolates FJ687177, FJ687179, FJ687189 and FJ687192 whose amino acids identities were less than 80 %. All the new CBSV isolates had limited amino acids identity (< 51.1 %) to SPMMV and TEV.

2. 4. 4 Foliage and root incidences and severity of CBSD

The mean field incidences and severities of CBSD on foliage and roots at different altitudes in the surveyed regions are indicated in Table 2. 3. The mean foliage incidence of CBSD ranged from 0 % to 84 % across the surveyed regions. No plant in Mbeya Region had foliage symptoms of CBSD. Highest incidences were observed in all regions of the Coastal Zone and the mean severity scale was 3.0. Five percent of assessed fields in the Coast and Tanga Regions (data not shown) had 100 % foliage incidences. The low altitude Islands of the Zanzibar archipelagos (below 40 masl) recorded the highest mean incidence and severity of the disease. In the Lake Zone (despite many sites being located above 1200 masl), 50 % CBSD incidence was associated with a severity score of 2.1. The mean foliage incidence of CBSD (51 %) in the Southern Zone (920-1800 masl) also suggested the presence of the disease across a wide range of altitudes.

Assessment of CBSD root symptoms showed that four of the twenty eight fields in the six Regions of the Coastal Zone had all roots affected. Tanga Region recorded the highest mean root incidence (82 %), associated with a mean severity of 4.3. An average CBSD root necrosis of 30 % associated with a mean severity of 2.3 was recorded in Mbeya, although none of the

assessed plants exhibited foliage symptoms. In Mara Region none of the plants (36 %) that exhibited CBSD shoot symptoms had root necroses.

Table 2.3 Field incidence and severity of CBSD and CMD at different altitude

Region	Fields assessed	Altitude (masl) [a]	CBSD incidence (foliage) [b]	CBSD severity (foliage) [c]	CBSD incidence (roots) [d]	CBSD severity (roots) [e]	CMD incidence (foliage) [f]
		Mean	Mean (range)	mean (range)	mean (range)	mean (range)	Mean (range)
Coast	5	209	75 (46.7 - 100)	3.1 (2.6 - 3.6)	10 (4 - 18)	2.3 (1 - 2.7)	39 (0 - 90)
Morogoro	1	541	50	2.7	N/A	N/A	7
Zanzibar archipelago	7	39	85 (13.3 - 100)	3 (2 - 3.5)	75 (0 - 100)	3.8 (1 - 4.8)	65 (7.1 - 100)
Tanga	5	560	56 (7.1 - 100)	2.8 (2 - 3.9)	82 (0 - 100)	4.3 (0 - 5)	41 (6.7 - 70)
Lindi	4	95	62 (27.7 - 80)	2.9 (2.4 - 3.3)	80 (0 - 100)	3.5 (1 - 4.2)	12 (6.7 - 20)
Mtwara	6	321	43 (6.7 - 93)	2.6 (2 - 3.4)	44 (0 - 100)	3.1 (1 - 4.8)	18 (3 - 50)
Mwanza	8	119	26 (0 – 63.3)	2.6 (1 - 2.8)	16 (9 - 20)	2.1(2 - 2.2)	37 (13 - 93.3)
Kagera	19	1307	26 (0 – 70)	2.3 (1 - 2.76)	42 (3.2 - 53.4)	2.3 (2 - 3.6)	39 (6.7 - 100)
Kigoma	5	1313	50 (0 – 66.7)	2.2 (1 - 2.5)	25 (0 - 25.2)	2.4 (0 - 2.4)	22 (13.3 - 43.3)
Mara	4	1265	37 (0 – 56.7)	2.4 (2 - 2.7)	0	1	28 (10 - 90)
Iringa	4	927	21 (0 – 50)	2.4 (1 - 2.9)	23 (15 - 33.3)	2.4 (2 - 2.8)	15 (0 - 26.7)
Mbeya	4	1017	0	1	30 (0 - 35.1)	2.3 (1 - 2.6)	52 (0 - 70)
Rukwa	2	1322	52 (50 - 53)	2.4 (2.3 - 2.4)	25 (0 - 25.2)	2 (1 - 2)	23 (0 - 23.3)
Ruvuma	17	947	36 (0 – 93.3)	2.5 (1 - 3.2)	39 (0 - 90.2)	2.8 (1 - 3.5)	29 (0 - 83)

Note: Numbers in brackets refers to the minimum through maximum value recorded.

[a]Average elevation for all the surveyed fields in each region.

[b]Percentage of total number of leaves with CBSD symptoms.

[c]Severity (mean) of CBSD foliage symptoms on 1 to 5 scale.

[d]Average of the percentage of total number of plants with CBSD symptoms in roots.

[e]Severity (mean) of CBSD symptomatic roots on 1 to 5 scale.

[f]Average of the percentage of total number of plants with CMD symptoms.

Generally, the Coastal Zone had greater CBSD foliage incidences compared to the Lake and Southern Zones (Figure 2.4). The disease parameters (Table 2.4) indicated that the Coastal Zone had the highest shoot and root incidence and severity of all the Zones. Foliage CBSD incidence was surprisingly low (27 %) in the Southern Zone compared to the root incidence (33 %). The country average suggested similar foliage (38 %) and root (36 %) incidences. The age of the assessed crops was about 8 months after planting in the Coastal and Lake Zones whilst in the Southern Zone the majority of the crop was approximately 12 months. These differences in crop age at time of sampling would have influenced the results for root necrosis.

Table 2.4 Disease, crop age and *B. tabaci* parameters for the three surveyed Zones

Diagnostic parameter	Coastal Zone (n =27)	Lake Zone (n = 36)	Southern Zone (n = 28)	Country Mean
Foliage CBSD Incidence (%)	62 (100)	26 (70)	27 (93.3)	38
Foliage CBSD severity	2.8 (3.9)	2.5 (2.8)	2.6 (3.3)	2.6
Stem CBSD Incidence (%)	35 (86.7)	1 (20)	6 (56.7)	14
Stem CBSD Severity	2.9 (4.5)	2.0 (2.0)	2.4 (2.6)	2.4
Root CBSD Incidence (%)	57 (100)	18 (53.4)	33 (90)	36
Root CBSD severity	3.5 (5)	2.5 (3.6)	2.7 (3.5)	2.9
Symptoms on leaf sample (%)	100	89	78	89
CBSV positive by RT-PCR (%)	79	72	70	74
Average crop age (months)	8	7	12	9
B. tabaci per plant	2	24	1	9
CMD incidence (foliage) (%)	32	35	24	31
CMD severity	2.9	2.9	2.6	2.8

Note; The maximum values recorded for each parameter is given in brackets.

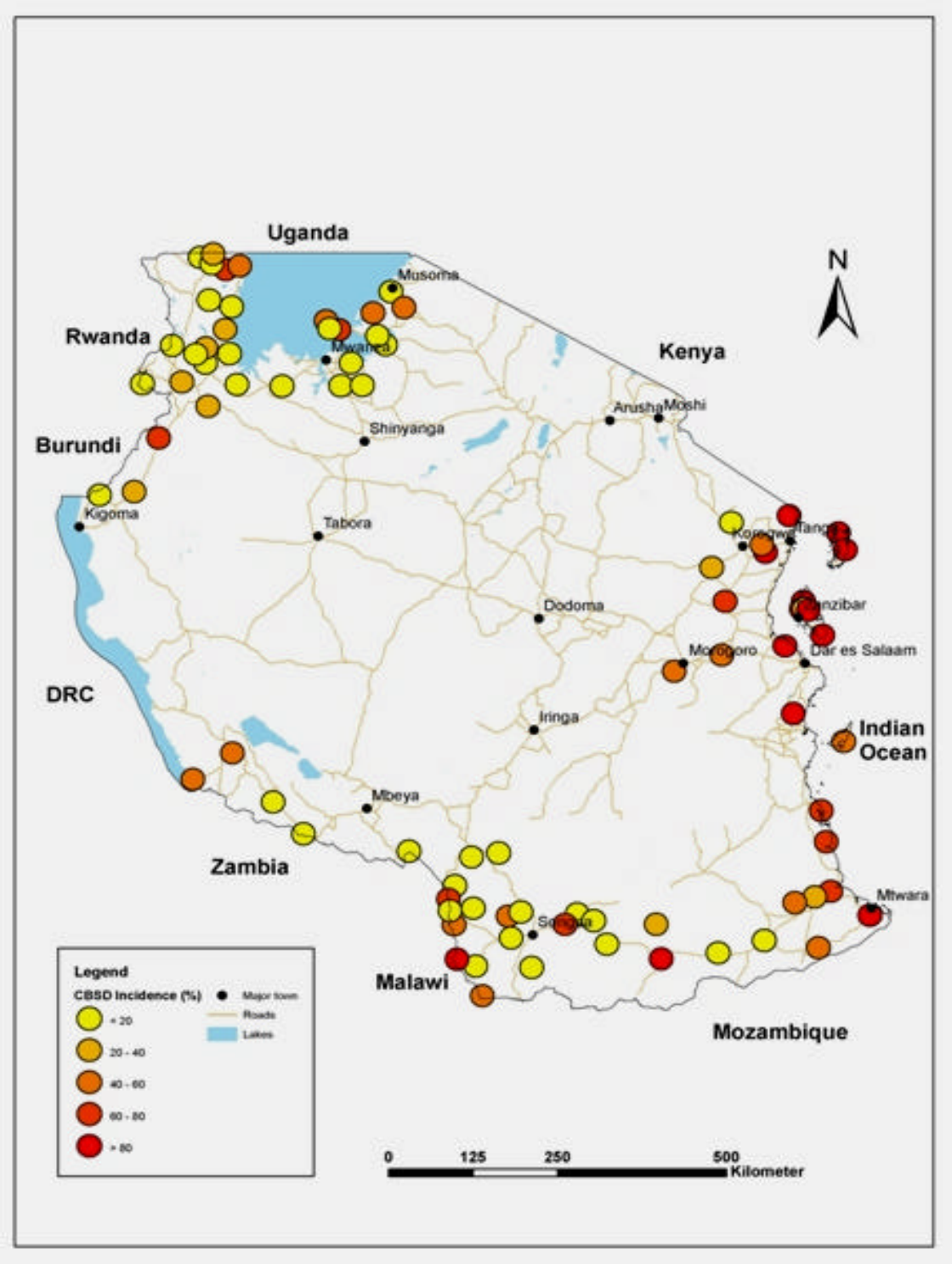


Figure 2.4 CBSD incidence of foliage symptoms in Tanzania

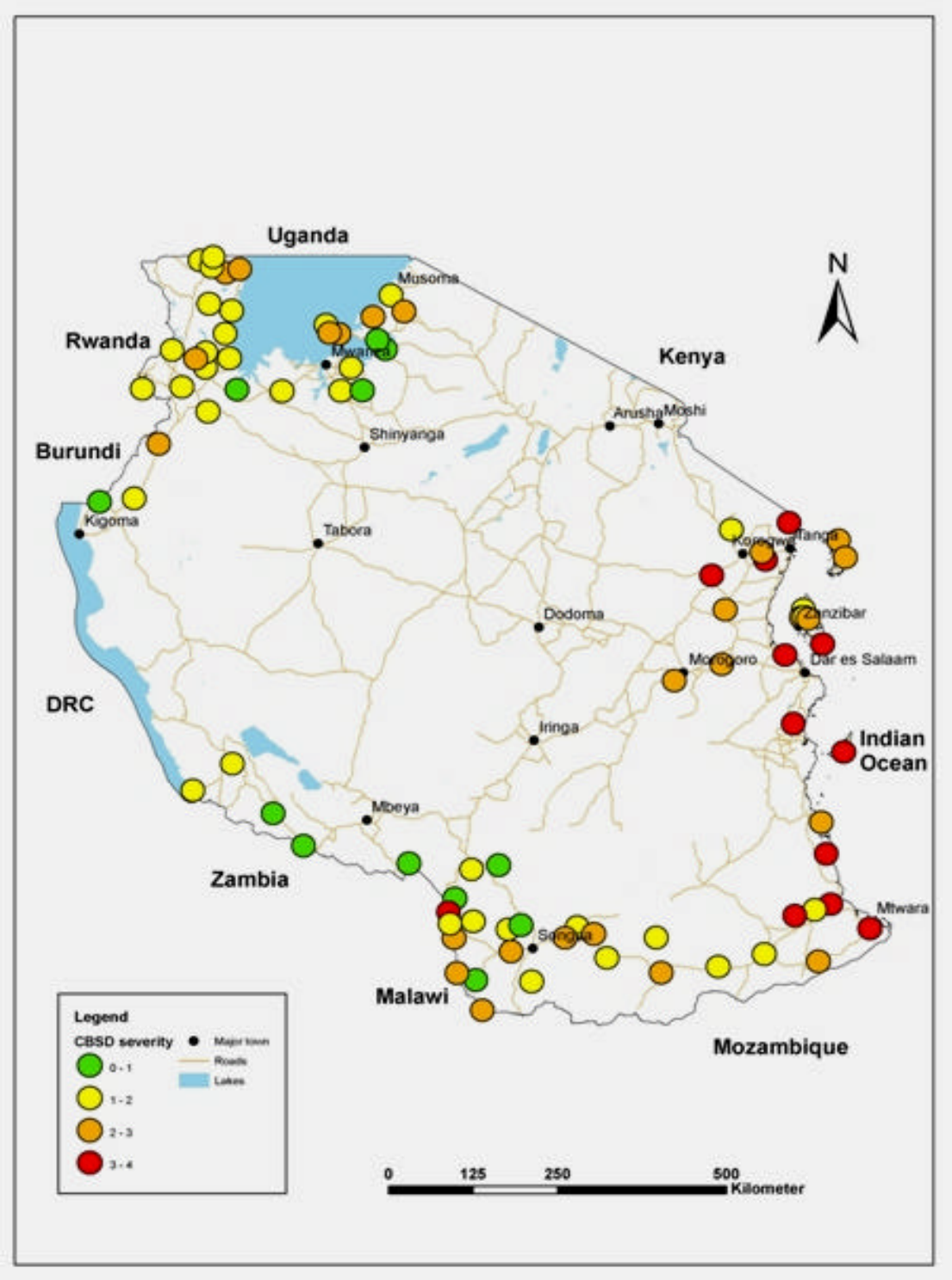


Figure 2.5 CBSD severity of foliage symptoms in Tanzania

The highest mean foliage severity (score 2.8) of CBSD was recorded in the Coastal Zone compared to the Lake and Southern Zones (Figure 2.5). CBSD foliage mean severities of 2.5 and 2.6 were recorded in the Lake and Southern Zones, respectively. The root severity scores were also highest in the Coastal Zone (3.5) compared to the 2.5 and 2.7 of the Lake and Southern Zones respectively.

2. 4. 5 Incidences and severity of CMD and whitefly numbers

CMD was recorded in 86 of 91 (95 %) surveyed sites (Table 2.4). The mean disease incidence was 31 %, the highest being in the Lake Zone (35 %). The lowest incidence was observed in Lindi Region (12 %) in the Coastal Zone. CMD severity was similar across the three surveyed Zones. The correlation analysis (Table 2.5) revealed no significant relationship between the disease parameters for CMD and those of CBSD.

The number of adult whitefly was very low across the surveyed sites except in the Lake Zone where the mean count of 24 adult whiteflies per shoot was recorded. Correlation analysis revealed a significant relationship between the number of whitefly and CMD incidences in all zones.

2. 4. 6 Correlation among disease parameters

The Pearson's linear correlation analysis of CBSD indices was as shown in Table 2.5. The correlation suggested a negative relationship between altitude and CBSD foliage, stem and root incidences and severities. That is, an increase in altitude led to decreased incidence and

severity of the disease. However, this influence of altitude on foliage, stem and root incidences and severity of CBSD was not statistically significant. Positive correlation existed between the incidence and severity of CBSD on foliage parts. The stem incidence had positive correlation with incidence and severity of CBSD on foliage and roots. Like CBSD, the incidence of CMD demonstrated a positive correlation with the severity of the disease.

Table 2.5 Correlation matrices between CBSD and CMD incidences and severities, *B. tabaci*, the crop age and field size in sites covered during the survey.

	FACTOR II											
FACTOR I	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	**K**	**L**	**M**
A: Altitude (masl)	1.00											
B: *B. tabaci*	0.12	1.00										
C: CBSD leaf inc. %	**-0.6**	-0.01	1.00									
D: CBSD leaf severity	**-0.55**	-0.02	**0.76**	1.00								
E: CBSD root inc. %	-0.42	-0.07	0.4	0.42	1.00							
F: CBSD root severity	-0.33	-0.05	0.35	0.39	**0.91**	1.00						
G: CBSD stem inc. %	**-0.65**	-0.09	**0.74**	**0.64**	**0.55**	**0.5**	1.00					
H: CBSD stem severity	**-0.68**	-0.1	**0.67**	**0.66**	**0.57**	**0.52**	**0.87**	1.00				
J: CMD inc. %	-0.08	0.16	0.06	-0.04	0.07	0.09	0.16	0.03	1.00			
K: CMD severity	-0.05	0.09	0.06	0.16	0.06	0.14	0.16	0.12	**0.51**	1.00		
L: Crop age (months)	0.12	-0.1	-0.14	-0.08	-0.03	-0.01	-0.09	-0.16	0.01	-0.01	1.00	
M: Field size (acre)	0.13	-0.01	0.05	0.01	0.01	0.05	-0.01	-0.04	-0.2	-0.1	0.05	1.00

2. 5 DISCUSSION

Results from this study have shown that CBSV and the disease that it causes occur throughout Tanzania wherever cassava is grown. Although previous studies (Hillocks *et al.*, 1996; Hillocks *et al.*, 1999; Legg and Raya, 1998; Nichols, 1950a; Thresh and Mbwana, 1998)

reported the restricted distribution of CBSD to coastal areas including Zanzibar, with isolated spots in Tabora Region and along the shore of Lake Malawi (Legg and Raya, 1998), the current findings revealed the widespread occurrence of the disease at mid-altitudes in the interior of the country. For the first time, CBSD was observed and the presence of CBSV confirmed in the Lake Zone of Tanzania.

The presence of CBSD in the Lake Zone has a major implication on food security. Based on existing statistics, the Lake Zone ranks second in cassava production in Tanzania after the Coastal Zone (Mkamilo, 2005). However, the few CBSD-resistant cultivars that are available in the Coastal Zone are still being tested and none has yet been confirmed to resist the disease in the Lake Zone (E. Kanju, pers. comm.). Therefore, with the observed CBSD incidence, the Lake Zone might suffer heavily from the impact of the disease. An added threat to food security in the Lake Zone is the presence of the cassava mosaic disease (CMD) pandemic (Legg *et al.*, 2006). Given the occurrence of the two virus diseases in largely susceptible cassava cultivars, the increased crop loss is imminent and hence there is a very uncertain food security situation in the Zone.

Other parts of the country, including the Southern Zone, were also affected by CBSD. Seventy percent of the samples collected were diagnosed positive for the virus in the Southern Zone. It suggests that CBSV is a problem throughout the cassava production systems in the country. Although widely distributed, the occurrence of CBSD in a few fields was in isolation, restricted to a single or few plants, particularly in the Southern Zones. In some fields, only 1 or a maximum of 3 of the 30 plants examined were affected in isolation by CBSD but with no

indication of a continuous spread of the disease in the neighbouring plants. A similar situation for CBSD incidence was reported previously by other workers (Legg and Raya, 1998). It is suspected that the recorded low incidences in these fields were due to the planting of diseased cuttings. This supposition is also based on very low average numbers of whitefly (one adult per plant) being recorded in the Zone.

Sequence variation was observed among the 43 CBSV-isolates (Rwegasira *et al.*, 2009) suggesting considerable diversity. The isolate, FJ687177 which claded differently from the six previously reported ones (Monger *et al.*, 2001b), could be a potentially new and distinct CBSV strain in Tanzania. This isolate had a closer relationship with the reported Ugandan isolates (Alicai *et al*., 2007). Similar observation on the close relationship among isolates from different countries was reported by Winter *et al.* (2008), in which the full genome sequence of CBSV isolates from Kenya, Malawi and Uganda were closely related by 95 % nucleotide identity. In addition, there were fourteen new CBSV isolates from Tanzania, which had nucleotide identity (< 80 %) with the three Ugandan isolates (Alicai *et al*., 2007) although they had closer identity (> 80 %) with the six Mozambican and Tanzanian isolates (Monger *et al.*, 2001b). According to ICTV (2005), rules on Potyvirus taxonomy based on full CP-genes sequences, same species should maintain greater than 80 % nucleotide identity and whenever the CP-gene sequence identity is less than 80 % it warrants to a new potyvirus strain or species. This further suggested the possible diversity among CBSV isolates. Isolates in this group included; FJ687180, FJ687206, FJ687183, FJ687187, FJ687188, FJ687189, FJ687190, FJ687194, FJ687198, FJ687199, FJ687200, FJ687204, FJ687205 and KECBSV were from Tanzania and Kenya.

Despite the observed nucleotide variation among CBSV isolates, they were all similar in terms of amino acids (> 80 % identity). Translation of nucleotides to amino acids in which different combinations of triplet nucleotide may code for the same amino acid was established as the reason for the similarity of isolates at amino acids level although they were diverse at nucleotides level.

Since the amino acids sequence homologies for isolates obtained in this study were all > 80 %, and considering the limits set for delineating species in potyviruses (Adam *et al.*, 2005; ICTV, 2005), they all appear to be CBSV. However, a full CP sequence would be required for a more definitive conclusion.

The similarity of CBSV isolates across Tanzania may be due to the extensive exchange of cassava planting materials within and between the farming communities (Mkamilo, 2005). Since cassava is a vegetatively propagated crop, spread of the virus might have occurred through free exchange of CBSV-infected cuttings. The isolate FJ687177 that appeared different from the other 42 Tanzanian isolates could have emanated from a wild cassava plant. As suggested by Storey (1936), on the possible origin of CBSV which was believed to have indigenous hosts, it is most probable that the isolate FJ687177 also originated from other indigenous host plant species.

The plant age from which the sample was collected had no effect on CBSV detection by RT-PCR. However, the plant age influenced the severity of CBSD symptoms particularly those in the roots. Mature plants (= 1 year) generally had severe root necrosis compared to young ones.

Increasing root symptoms severity with plant age is a notable feature of CBSD (Hillocks *et al.*, 2001; Nichols, 1950a). Foliage symptoms were also observed to vary with plant age. In the Southern zone, plants aged = 8 months had shed the lower first-formed leaves making it difficult to observe the foliage symptoms. Elsewhere it has been reported that, under these circumstances, it is difficult even for an experienced observer to positively diagnose CBSD-affected plants (Nichols, 1950a). Generally, the severity of symptoms increased with age although a few plants aged 4-months in some cultivars exhibited clear foliage symptoms of CBSD.

The mean foliage incidence of CBSD (62 %) recorded in the Coastal Zone was relatively high compared to 26 % and 27 % found in the Lake and Southern Zones, respectively. This is attributed to the suitable weather condition for CBSD development and the sensitivity of the locally grown cultivars that have been continuously recycled season after season in the Coastal Zone for more than seventy years (Storey, 1936). The recorded foliage incidence in the Lake and Southern Zones (> 1600 masl), where the disease has never been reported previously, suggests recent spread of CBSD into inland mid-altitude areas.

Considering disease indices in each region, the 84 % mean CBSD foliage incidence recorded in Zanzibar (altitude < 200 masl) was relatively higher compared to the 32 % previously reported in Zanzibar (Thresh and Mbwana, 1998), and 57 % reported at the similar elevation in Mtwara region (Hillocks *et al.*, 1999). This incidence reported in Mtwara was previously believed to be the highest in Tanzania. The general observation indicated that CBSD foliage incidence was increasing in all farmer-grown cultivars, particularly in the Coastal Zone.

In the Southern Zone, CBSD incidence was more apparent in the roots (33 %) than the foliage parts (27 %). This observation suggests the possible influence of weather and plant age on CBSD symptoms expression. Mature cassava plants tend to shed most symptomatic leaves when the warmer moist season succeeds the cooler dry season (Hillocks, 1997; Jennings, 1957; Nichols, 1950a). This results in the roots being the only indicators for CBSD. From the observations made during this study, most of the assessed fields in the Southern Zone were >12 months in age. In contrast to these observations, the disease incidence in the Coastal and Lake Zones where the assessed plants were aged between 3 and 9 months, more CBSD foliage incidences of 62 % and 26 % were recorded compared to the root incidences of 57 % and 18 % respectively.

CBSD incidences were relatively low in stems compared to the foliage and root incidences in all Zones. The mean stem incidence of 35 % in the Coastal Zone, 1 % in the Lake Zone and 6 % in the Southern Zone suggest that stem symptoms alone are not good indicator of the disease. This concurs with observation by other workers (Nichols, 1950a) that stem lesions are not the most characteristic symptom of CBSD.

Pearson's correlation analysis suggested a positive correlation between root, stem and foliage incidences and severity of CBSD, although this was not statistically significant. The positive correlation between shoot and root severity was recorded where CBSD-affected plants exhibited stem die-back (data not shown). Similar observation was reported previously (Hillocks *et al*., 1996). The correlation between shoot and root incidences and severity of CBSD were

inconsistent at two sites; Nduna, (Ludewa) and Muheza, (Tanga), where plants without foliage or stem symptoms had necrotic roots.

The current study has for the first time identified and confirmed the occurrence of CBSV and CBSD in areas of Tanzania not reported before, particularly the Lake Zone. The majority of local cultivars are susceptible to the disease. Most CBSV isolates are similar in amino acids sequence comparisons, although there are some variations, which suggest strain diversity. The finding in this study confirms results obtained from mid-altitude areas of Uganda (Alicai *et al*., 2007) that CBSD is not constrained to altitude below 1000 masl as previously suggested. The incidence and severity levels of the disease indicate a potentially new epidemic in the Lake Zone, which may exacerbate the food shortages already caused by the CMD pandemic. In view of the findings in this study, further similar work in neighbouring countries particularly Burundi, DRC and Rwanda is warranted in order to gauge the full extent of CBSV occurrence in East African region.

CHAPTER 3

EFFICIENCY OF NON-VECTOR METHODS IN TRANSMISSION OF *CASSAVA BROWN STREAK VIRUS* (FAMILY *POTYVIRIDAE:* GENUS *IPOMOVIRUS*) TO SUSCEPTIBLE CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

Manuscript in preparation for submission to Pest Management Journal

3. 1 SUMMARY

Transmission through *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae) and dissemination of virus-infected cuttings are the reported mechanisms through which *Cassava brown streak virus* (CBSV) is spread. In the current study, CBSV was successfully transmitted through a series of non-vector techniques. Subsequent detection and confirmation of CBSV infections were done by RT-PCR using coat protein gene-specific CBSV primers.

In replicated screen-house experiments, transmission of CBSV was achieved through cutting tools (22 %) using susceptible cassava cv. Albert as test plants. Up to 54 % transmission efficiency was achieved through sap inoculation of CBSV from infected cassava to CBSV-free plants cv. Mreteta. Grafting CBSV-free susceptible scions onto CBSV-infected rootstocks was the most efficient CBSV transmission technique and up to 100 % of scions were infected within 4-weeks. The infected plants developed characteristic foliage vein chlorosis and blotches on the previously symptomless CBSV-free scions. The virus was not transmitted from infected root debris to cassava seedlings or virus-free cuttings.

For the first time, the efficiency of graft transmission of CBSV in cassava was determined and the best graft combination established. CBSV transmission by cutting tools was also established for the first time. The findings suggest that the non-vector methods could contribute more to CBSV spread than the whitefly, *B. tabaci*.

3. 2 INTRODUCTION

Since the first report (Storey, 1936), CBSD was known to be most prevalent in coastal East Africa below 500 metres above sea level (masl) (Nichols, 1950a). Until recently, it was rarely observed above 1000 masl (Hillocks, 1997; Hillocks *et al.*, 1996; Jennings, 1957; Legg and Raya, 1998). Recent observations (Chapter 2) have indicated the widespread occurrence of CBSD in all cassava growing areas in Tanzania regardless of altitude. In addition, a new outbreak was reported from parts of Uganda all above 1000 masl (Alicai *et al.*, 2007). The cause for such a big increase in disease prevalence is not known.

Transmission of CBSV from plant to plant is reported to occur through grafting CBSV-free with infected cutting (Lister, 1959; Nichols, 1950a; Storey, 1936, 1939) and subsequent dissemination by infected cuttings. However, natural spread of CBSD to originally uninfected cassava has been reported (Hillocks *et al.*, 1999; Kanju *et al.*, 2003). Suspected transmission of CBSV by the whitefly vector, *B. tabaci* (Storey, 1939) was confirmed by Maruthi *et al.* (2005) although at a low rate *ca* 2 %. Previous attempts to transmit the virus by the aphid, *Myzus persicae* Sulz (Hemiptera: *Aphididae*) and other aphid species failed (Bock, 1994; Lennon *et al.*, 1986).

Since vector transmission was proven inefficient, the non-vector mechanisms were suspected to contribute to the spread of the virus. Lister (1959) reported success with artificial sap transmission of CBSV, although the infection rate was not stated. Moreover, the reported graft transmission of the virus (Storey, 1936; 1939) did not examine the efficiency of the

technique. The time taken for symptoms to appear when the infected and non-infected parts of the plants are used interchangeably as scion and rootstock was not determined. Other non-vector methods of CBSV transmission including seed, cutting tools, leaf harvesting and CBSV-infected root debris (crop residues) have not previously been explored.

The current studies aimed at identifying the non-vector mechanisms of CBSV transmission and assessing their effectiveness and efficiency. The specific objectives of the study were; (i) to determine the possible role of cutting tools and leaf harvesting; (ii) to determine the efficiency of sap and graft-based transmission; (iii) to explore the comparative effect of using infected and non-infected scions and rootstocks in grafting; (iv) to establish if CBSV-infected root debris might cause new infection, and (iv) to determine if CBSV is transmitted through seeds of infected plants.

3. 3 MATERIALS AND METHODS

A series of experiments was conducted in the screenhouse. CBSV-free plants for experiments were generated by meristem tissue culture and a few were obtained as in-vitro cultures from IITA. All tissues were tested in triplicate by reverse transcriptase polymerase chain reaction (RT-PCR) using CBSV CP gene-specific primers (CBSV10 and CBSV11) (Monger *et al.,* 2001a). The CBSV transmission experiments included; sap, grafting, seeds, CBSV-infected cassava root debris, leaf harvesting and cutting tools. All experiments were replicated (three to six times) and controls were included.

3. 3. 1 Generation of CBSV-free cassava plants and molecular indexing for the virus

Cuttings from symptomless cassava plants cvs. Albert, Mreteta, Namikonga and Supa were collected from experimental plots at Naliendele Research Institute, Mtwara and pre-sprouted at a nursery unit at the Sugarcane Research Institute (SRI), Kibaha in Tanzania. Plant shoot tips were harvested and used for meristem tissue culture (George, 1993; Wang and Hu, 1980) at Mikocheni Agricultural Research Institute (MARI) in Dar es Salaam. Dr. I. Ingelbrecht of IITA, Ibadan, kindly supplied CBSV-free tissue culture plantlets cv. Albert for experimental purposes. Triplicate RT-PCR tests using coat protein gene-specific primers for CBSV (Monger *et al.,* 2001a) were done on the tissue culture plants (molecular indexing). Only plants proven to be virus-free were used for experimentation.

3. 3. 2 Sap inoculation experiments

Sap transmission experiments (Plate 3.1) were conducted in the screenhouse at MARI from March to December, 2007. Two formulations of inoculation buffer (annotated as A and B) were prepared and used to sap-inoculate CBSV-free tissue culture-generated plants of cv. Mreteta. Buffer A comprised 0.05 M monobasic potassium phosphate (KH_2PO4), 50 mM glycine ($C_2H_5NO_2$), 1 % bentonite and 1 % celite (SiO_2), suspended in double-distilled water, adjusted to pH 9.0, the volume made to 100 mls and autoclaved. Buffer B comprised 0.1M solution of monobasic potassium phosphate (KH_2PO4) mixed with 1 % celite (SiO_2) in double-distilled water, adjusted to pH 7.5, the volume made to 100 mls with distilled water and autoclaved. The control leaf samples were collected pre-inoculation from each test plant. The youngest symptomatic leaves collected from CBSV-infected plants cv. Albert were ground in a

pre-chilled mortar and pestle with each of the inoculation buffers at 1:1 ratio (w/v) and inoculated carefully onto the second top (fully open) leaf of the test plant. The mortar and pestles were disinfected and treated with diethyl pyrocarbonate (DEPC) water prior to use. Inoculation assays were done in triplicate with each treatment comprised of eight plants. Samples were collected from a young top leaf for virus detection by RT-PCR after 2, 3 and 5 days and thereafter weekly up to 24 weeks. Collected samples were stored at -80^{0}C prior to CBSV testing.

3. 3. 3 Graft inoculation

Graft transmission experiments (Plate 3.2) were conducted in a screenhouse at MARI from June to December, 2007. Two graft options were made in which the CBSV-infected and CBSV-free seedlings were used as either scion or rootstock. A modified cleft/wedge grafting technique was used as described previously (Anon, 1993; Rothenberger and Starbuck, 2002). The virus-indexed pre-sprouted plants of the cv. Albert developed by tissue culture and pre-germinated infected cuttings from diseased plants of the same variety were used. The former were developed in the laboratory and plantlets acclimatized in the screenhouse while the latter were obtained from the field and pre-sprouted in pots in the screenhouse. Grafting was done when the stem girth at 15 cm from the soil level was at least 10 mm. The graft portions were tightly taped with parafilm to promote union and prevent desiccation. The grafted plants were protected from excessive evaporation by a plastic bag cover which was removed after 4 days. Grafted plants were maintained in the screenhouse and monitored for symptom expression and samples were collected regularly for post-inoculation detection of CBSV. In the infected rootstock-healthy scion treatment, the samples were collected from the top leaf lobe after 2, 3 and 5 days and thereafter weekly up to 24 weeks and stored at -80^{0}C for CBSV detection by RT-

PCR. In the infected scion-healthy rootstock treatment, leaf lobes below the graft union were collected at similar intervals.

3. 3. 4 Seed transmission of CBSV

The seed transmission experiment was conducted at SRI, Kibaha, in Tanzania. Seeds were collected from six CBSV-infected local cassava cvs. Albert, Limbanga Red, Limbanga White, Mreteta, Sharia and Supa in farmers' fields in Mtwara Region. Leaf samples were collected from each of the source plants for confirmation of the presence of CBSV by RT-PCR using CP gene-specific primers at MARI. The seeds were surface sterilised with 75 % ethanol, 10 % sodium hypochlorite and distilled water then planted in heat-sterilised soil. Experimentation began in August, 2006 and ended in October, 2007. Two experiments were conducted, each for six months. Seeds of each cultivar were planted in six pots (six replications) and thinning of surplus seedlings done a week after germination (Plate 3.3). Five seedlings of each cultivar were maintained in each of six pots giving a total of 180 plants (6 varieties x 6 pots x 5 seedlings). Leaf samples for CBSV detection were collected starting at two weeks (at three fully open leaves) after germination, and thereafter monthly, for six months. Samples from each pot were bulked into one sample for RT-PCR.

3. 3. 5 Effect of CBSV-infected root debris on seeds and CBSV-free cuttings

Two different experiments of similar set up were conducted from July, 2007 to January, 2008 at SRI, Kibaha to assess whether infected cassava debris might be a source of new infection of CBSV. In the first set of experiments, the roots from infected plants cv. Cheupe were dug up, chopped into small pieces and mixed with sterile soil at ratios (debris:soil) of 0:4, 1:3 and 2:2

(v/v). These ratios were determined in a preliminary experiment using a ratio of 3:1 (debris:soil), in which none of the planted seeds germinated. Water was applied to the soil-plant debris mixture in 10 litre volume pots to field capacity and six surface-sterilised seeds cv. Albert were planted per pot. Each treatment was replicated thrice to give a total of 54 test plants (3 ratios x 6 seeds x 3 replications). Leaf samples were collected monthly for CBSV detection. The duration of the experiment was four months.

A similar experiment was set up in the same screenhouse at SRI, Kibaha which involved soaking CBSV-free cassava cuttings cv. Albert in distilled water-root debris mixture at varied volumes ratios. Distilled water and chopped root debris from CBSV-infected plants cv. Cheupe were mixed at ratios 0:4, 1:3, 2:2 and 3:1 (v/v of debris by water). The soaked cuttings were retrieved from the debris-water mixture after 6-12 hours and planted in potted sterile soil (Plate 3.4), three cuttings per pot. Ten litre capacity plastic pots were used in this experiment.

3. 3. 6 Effect of leaf-harvesting

An experiment to test the possibility of CBSV spread through leaf harvesting as commonly done by those who use cassava leaves as a vegetable, was conducted in the screenhouse (Plate 3.5) at SRI, Kibaha from September, 2007 to March, 2008. Fifteen CBSV-free plants cv. Albert were grown in pots alongside five CBSV-infected cuttings of the same variety. At five months of age, CBSV transmission by leaf harvesting was attempted. A leaf was harvested from an infected plant and then the same hand was used to harvest leaves from three CBSV-free plants. Leaf harvesting was done for all fifteen initially CBSV-free plants on a

monthly basis for four months. Leaf samples were collected monthly for CBSV detection by RT-PCR. CBSD symptoms expression was also monitored and recorded each month.



Plates 3.1-6, non-vector transmission of CBSV. **Plate 1,** Sap inoculation of CBSV with buffers A and B. (Buffer A contained 0.05 M monobasic potassium phosphate, 50 mM glycine, 1 % bentonite and 1 % celite in distilled water at pH 9.0. Buffer B contained 0.1M monobasic potassium phosphate and 1 % celite in distilled water at pH 7.5). **Plate 2.** Graft inoculation with CBSV-infected and CBSV-free scion and rootstocks cv. Albert. **Plate 3.** Seedlings from CBSV-infected mother plants. **Plate 4.** CBSV infected root debris as used in 3.3.5. **Plate 5.** CBSV transmission by leaf harvesting. **Plate 6.** Plants stems chopped down using a knife (cutting tools) initially cut through an infected stem.

3. 3. 7 Effect of cutting tools

A pot experiment to assess cutting tools (knives) as a possible means of transmission of CBSV was conducted in the screenhouse at SRI-Kibaha from January, 2007 to March, 2008. Ten CBSV-infected cuttings cv. Albert were established individually in pots and thirty CBSV-free cuttings (ten for each of the cvs. Albert, Mreteta and Supa) were also planted individually in pots. After six months a knife used to cut through an infected stem cv. Albert was used immediately afterwards to cut through CBSV-free cutting of cvs. Albert, Mreteta and Supa. (the CBSV-free plants were previously developed through tissue culture and maintained in the screenhouse at SRI, Kibaha). A single cut through an infected stem was followed by a cut on each of three different CBSV-free stems (Plate 3.6). Three treatments were made per variety in a three replicate experiment to give a total of 27 inoculated plants (3 plants x 3 varieties x 3 replications). One plant of each cultivar was maintained as a control i.e was not inoculated through cut. The experimental plants were 30 in total, (27 cut-inoculated and 3 controls). Leaf samples were collected monthly starting at three months after sprouting (post-inoculative cuts) for CBSV detection. Sample collection for detection of the virus continued for six months.

3. 3. 8 Isolation of RNA

Total RNA was extracted from 0.1g fresh leaf tissue in 4 M guanidium thiocyanate (Sigma, 59980) buffer mixed with 2-mercaptoethanol (Sigma, M3148) at a ratio of 1:125 (2-mercaptoethanol to guanidium) using a sterile mortar and pestle. Five hundred µl of the lysate were transferred to a 1.5 ml ependorf tube, 500 µl of 2.0 M sodium acetate added and the tube contents were thoroughly mixed. The tube content was added with 400 µl of 24:1 [chloroform ($CHCl_3$) to isoamyl alcohol (C_5H_2O)], mixed and incubated on ice for 10 minutes. Tubes were

centrifuged at 13000 rpm for 15 minutes and 450 µl of supernatant transferred to new tubes. Five hundred µl of ice-cold isopropanol was added to precipitate the RNA and the tube was then incubated at –20 ^{0}C for 10 minutes. The chilled contents were centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. The RNA pellet was air-dried after washing with 500 µl of 75 % ethanol and centrifuging at 13000 rpm for 2 minutes. Thirty five µl of RNase free water were added to dissolve the RNA pellet for analysis in RT-PCR.

3. 3. 9 Nucleic Acid Amplification by RT-PCR.

Triplicate RT-PCR was performed in a one-step reaction using superscript ^{TM} III RT/Platinum® *Taq* Mix System (Invitrogen Life Technologies) using a GeneAmp PCR system 9700 (Applied Biosystems, UK). The primers, CBSV 10F and 11R (Monger *et al.*, 2001a) designed to amplify an approximately 231 bp segment of the CP gene were used. Each 50 µl reaction mixture comprised 25 µl of 2X reaction mix (buffer with 0.4 mM of each of dNTP, 3.2 mM $MgSO_4$ and stabilizers), 1 µl of RNA, 0.4 µl of each of the forward and reverse primers, 1 µl of superscript RT-Taq (Invitrogen 12574-026) and 22.2 µl of sterile water. The PCR conditions were as follows; Initial cDNA synthesis and denaturation; at 55°C for 0.5 min. and 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min (in 35 cycles) followed by final extension at 72°C for 10 min.

3. 3. 10 Analysis of RT-PCR product.

RT-PCR products were separated electrophoretically in a 1.2 % agarose gels in 0.5X Tris Acetate EDTA (TAE) buffer, for 1 hour at 92 volts. Amplicons were visualised by staining with

ethidium bromide (0.01 µl/ml) under ultraviolet (UV) light and recorded using an image analyser (Syngene).

3. 4 RESULTS

Data obtained suggest that CBSV may be transmitted through a number of non-vector mechanisms (Table 3.1). Sap inoculation, grafting, leaf harvesting and cutting tools contributed appreciably to new infections of CBSV in CBSV-free plants. Contrastingly, the virus was not transmitted through seeds germinated from CBSV-infected plants or by cassava root debris of the CBSV-infected plants.

3. 4. 1 Sap transmission of CBSV

A period of 1–4 months was required before the virus could be detected in the sap-inoculated plants. A total of 13 of 24 (54 %) of plants inoculated using buffer B tested positive for CBSV compared to the 7 of 24 (29 %) which tested positive when inoculated with the more complex buffer A. An average of 27 days after inoculation (DAI) was required to the first detection of CBSV in plant tissues when either buffer A or B was used for inoculation. Plants inoculated using buffer B required fewer days (28 DAI) to first appearance of CBSD symptoms compared to (30 DAI) when buffer A was used. The proportion of plants that developed CBSD symptoms was 33 % with buffer B and only 16 % with buffer A.

Table 3.1 Summary of non-vector transmission and infectivity of CBSV

Treatment	Replicates	Time to CBSV detection	Time to symptoms	CBSV Positive (RT-PCR)	Number symptomatic
Sap inoculation					
Buffer A	3	28 - 122 DAI	30 - 35 DAI	7/24	4
control	3	0	0	0/3	0
Buffer B	3	27 - 124 DAI	28 - 68 DAI	13/24	8
Control	3	0	0	0/3	0
Grafting (scion)	N/A	26 DAI	28 DAI	10/10	10
Control	N/A	0	0	0/2	0
Grafting (rootstock)	N/A	12 DAI	20 DAI	10/10	10
Control	N/A	0	0	0/2	0
Seeds from infected	6	0	0	0/180	0
Seeds on debris:soil					
ratio 0:4	3	0	0	0/15	0
ratio1:3	3	0	0	0/15	0
ratio 2:2	3	0	0	0/15	0
Cuttings on debris:soil					
ratio 0:4	3	0	0	0/12	0
ratio1:3	3	0	5 months	0/12	1*
ratio 2:2	3	0	6 months	0/12	3*
ratio 3:1	3	N/A	N/A	N/A	N/A
Cuttings pre-soaked (debris:sterile water)					
ratio 0:4	N/A	0	0	0/4	0
ratio1:3	N/A	N/A	N/A	N/A	N/A
ratio 2:2	N/A	N/A	N/A	N/A	N/A
ratio 3:1	N/A	N/A	N/A	N/A	N/A
Leaf harvesting	3	74 DAI	110 DAI	1/15	1
Control	N/A	0	0	0/2	0
Cutting tools	3	113 DAI	132 DAI	6/27	2
Control	N/A	0	0	0/3	0

* CBSD-like symptoms but not confirmed by RT-PCR. Control refers to the plants to which the respective treatments were not applied. DAI; days after inoculation, N/A; not applicable

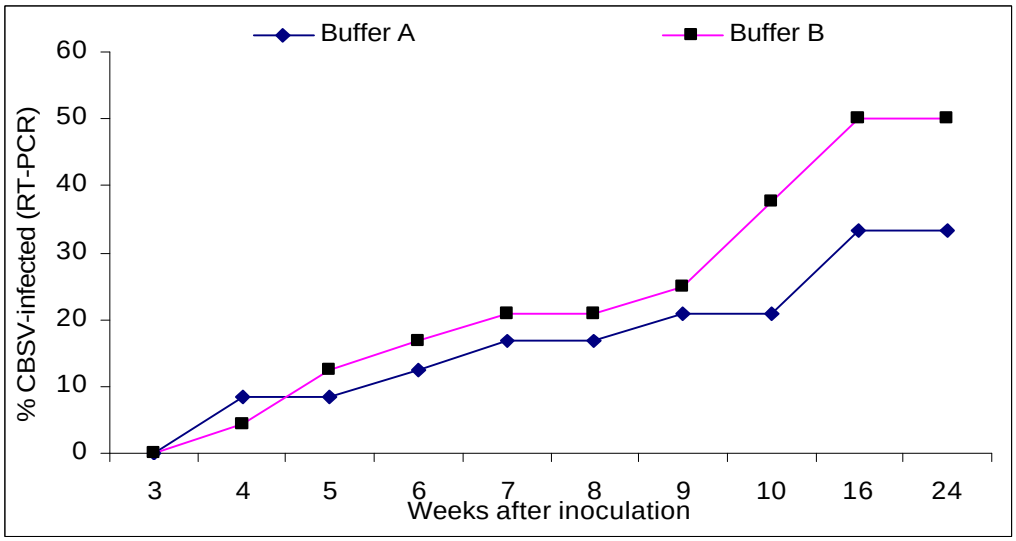


Figure 3.1a Relationship between inoculation buffers and time to CBSV detection in leaf tissues of the inoculated plants of cv. Mreteta.

Although more plants *ca* 8 % were positive with buffer A compared to *ca* 4 % with buffer B, 4-weeks after inoculation, (Figure 3.1a) more infections were increasingly recorded with buffer B than buffer A. The superiority of buffer B was similarly observed in the rate of symptoms expression with more than 33 % compared to the *ca.* 16 % with buffer A (Figure 3.1b). The decline in percentage of symptomatic plants ten weeks after inoculation with buffer A was caused by reduced number of older symptomatic leaves due to senescence.

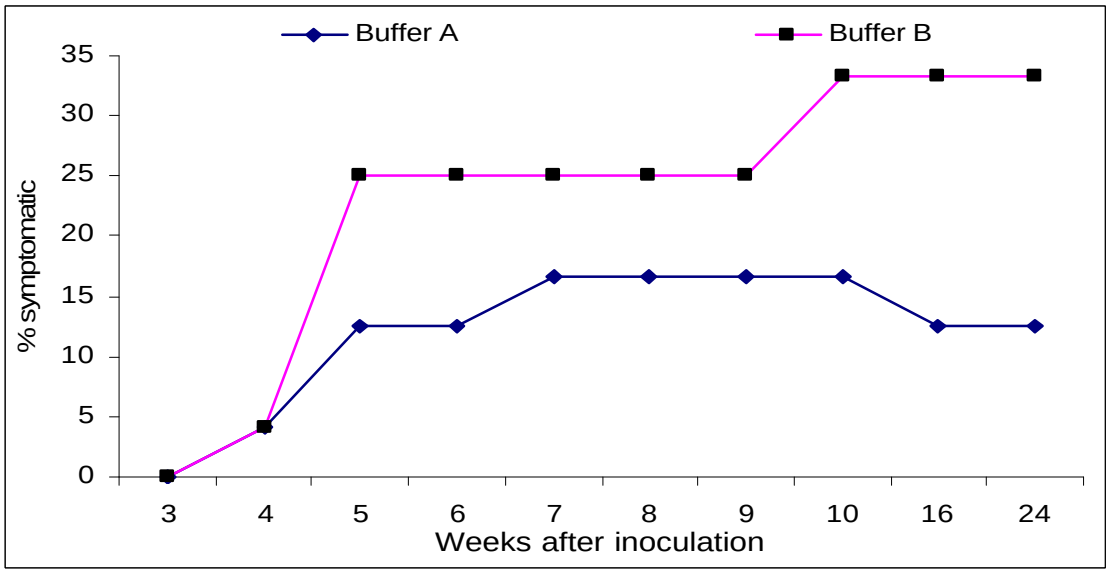


Figure 3.1b Rate of symptom development with inoculation buffers A and B used to infect CBSV-free tissue culture plants using tissues from CBSV-infected plants.

3. 4. 2 Graft transmission of CBSV

In the graft transmission experiments, 12–26 days were required to attain infection of all the test stock, suggesting that the method is the most efficient with respect to the rate of transmission and time. Initially, CBSV-free susceptible plants developed characteristic leaf vein chlorosis and necrotic blotches typical of CBSD in less than one month after graft inoculation. Earlier detection of the virus *ca* 12 DAI was recorded in grafts that involved infected rootstock and virus-free scion compared to the reciprocal combination (Table 3.1). Likewise, less time was required for symptom expression, 20 DAI compared to the 28 DAI for the infected scion to virus-free rootstock. Short periods of *ca* 4-weeks were required to attain 100 % incidence in the infected rootstocks grafted to the virus-free scions compared to the five weeks in the virus-free rootstocks grafted to the infected scions (Figure 3.2a).

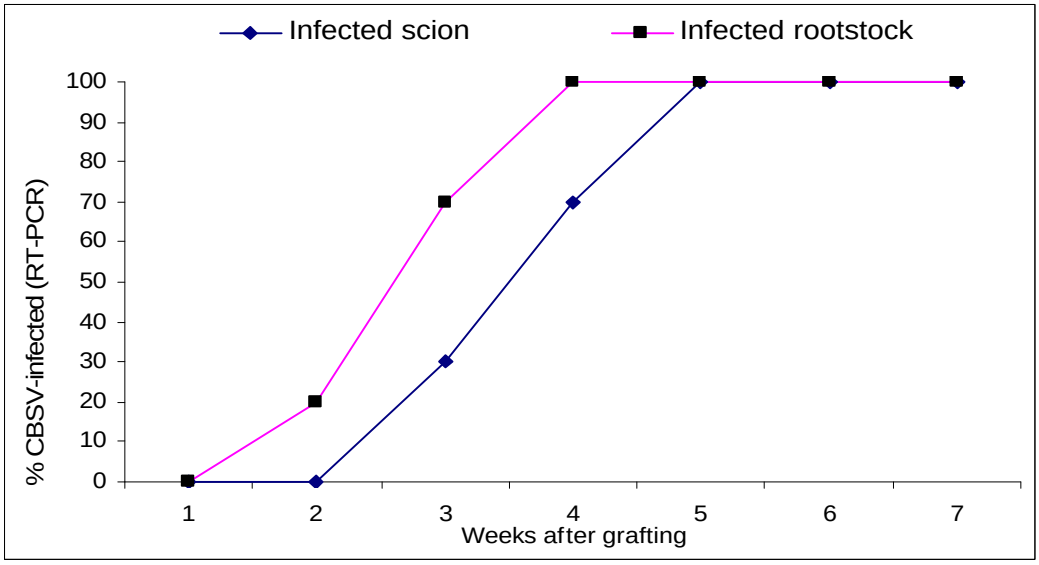


Figure 3.2a Relationship between graft methods and time to CBSV detection in cv. Albert.

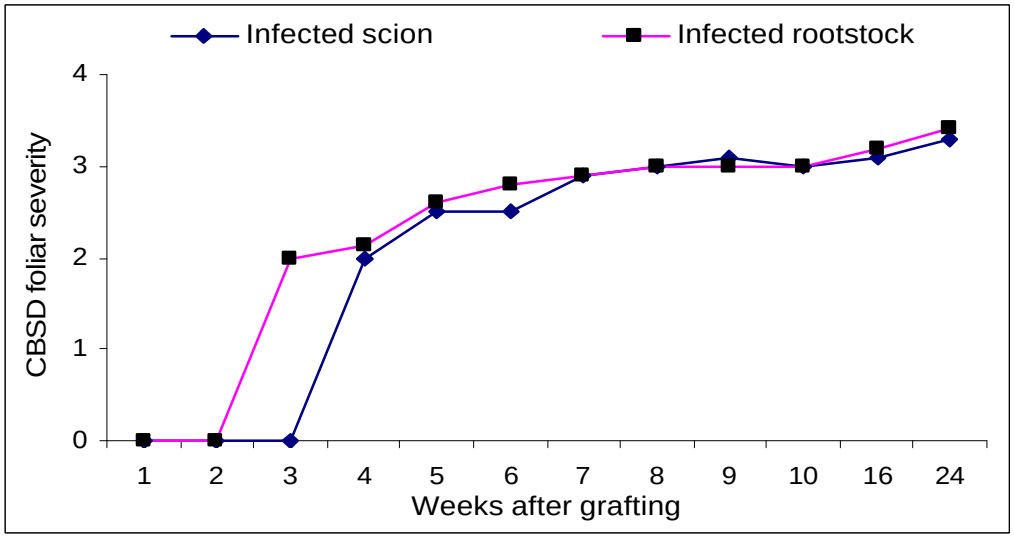
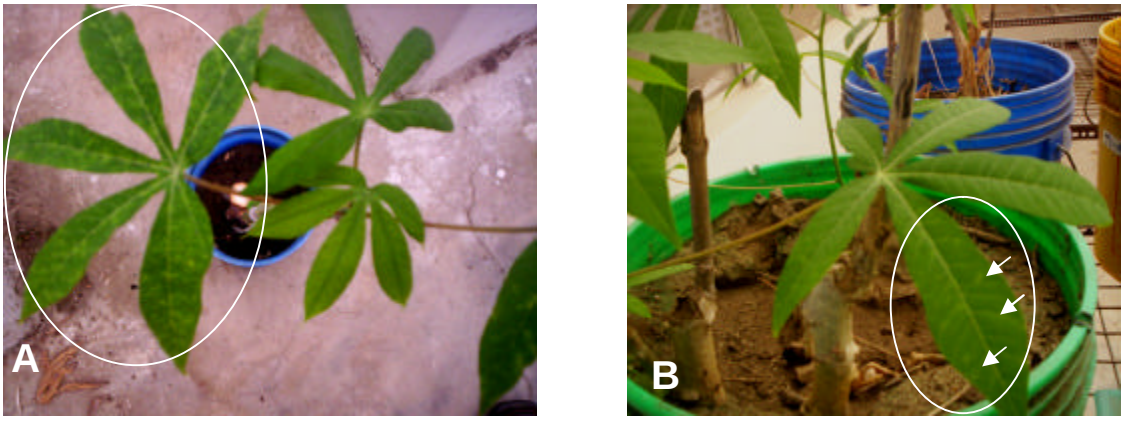


Figure 3.2b The relationship between mean foliage severity of CBSD and time after grafting in the two graft methods in cv. Albert.

Generally, the experimental results suggested that a maximum of five weeks were required to attain 100 % incidence with either of the graft techniques used. Leaf symptoms were recorded earlier (two weeks) in infected rootstock-virus free scion grafts compared to the reverse *ca* four weeks (Figure 3.2b). The observed leaf symptoms include necrotic blotches (Plate 3A) and feathery chloroses. Despite the variation in time to attain a severity score exceeding 2, symptoms gradually increased with time for both graft techniques.



Plates 3 A and B: Observable CBSV leaf symptoms from two CBSV transmission experiments; **A**, white circle indicating numerous chlorotic blotches on leaves in graft transmission with CBSV-free scion on CBSV-infected rootstock; **B**, brown chlorotic spots (shown by white arrows in the white circle) on the leaf of plant infected through cutting tools.

3. 4. 3 CBSV transmission by seeds

None of the seedlings developed from seeds of CBSD affected mother plants exhibited CBSD symptoms. All plants tested negative in RT-PCR even after repeated tests for more than six months. CBSV was never detected even after repeated experimentation.

3. 4. 4 CBSV transmissions to seeds and cuttings by infected debris

None of the seedlings which were sown in root debris from CBSV-infected plants became infected. Neither CBSD symptoms nor virus detection by RT-PCR signified the presence of the virus in the experimental plants. Although four of twenty four plants (17 %) at debris:soil ratios of 1:2 and 2:2, exhibited chlorotic leaf symptoms that were somewhat similar to those of CBSD, none of them tested positive to the virus even after repeated testing in a triplicate RT-PCR. Plants in the debris:soil ratio of 3:1 hardly sprouted and those that did died a week after sprouting.

In pre-soaking experiments that involved CBSV-infected root debris in water, none of the planted cuttings sprouted for any dilution ratio. The same results were recorded even after repetition of the experiment with reduced exposure of the cuttings (between six and twelve hours). Therefore, no infection data were obtained from these experiments.

3. 4. 5 CBSV transmission by cutting tools

Cutting tools infected 6 of 27 (22 %) test plants. Infections were confirmed by RT-PCR. However, only 2 of the 6 CBSV-positive plants developed disease symptoms (Plate 3B). Irregular-shaped cholotic spots were observed and later coalesced to form blotches. A relatively long time was required (113 DAI and 132 DAI) to first detection of the virus and development of CBSD symptoms, respectively.

3. 4. 6 CBSV transmission by leaf harvesting

One of 15 plants (7 %) in the leaf harvesting experiment became infected with the virus. The infected plant developed characteristic CBSD symptoms of feathery chlorosis which started in tertiary veins extending to cover the secondary and primary veins. The CBSD symptomatic plant tested positive with RT-PCR.

3. 4. 7 Confirmation of CBSV infection in inoculated tissues

The target band of about 231bp was amplified successfully in all infected samples using the CBSV coat protein gene-specific primers (Figure 3.3), thereby confirming the presence of the virus. Based on band strengths, the virus was detected strongly in graft transmission and in buffer B compared to the rest of the transmission methods tested. Despite the slight variation in band strength, the virus was detected positively in the infected samples.

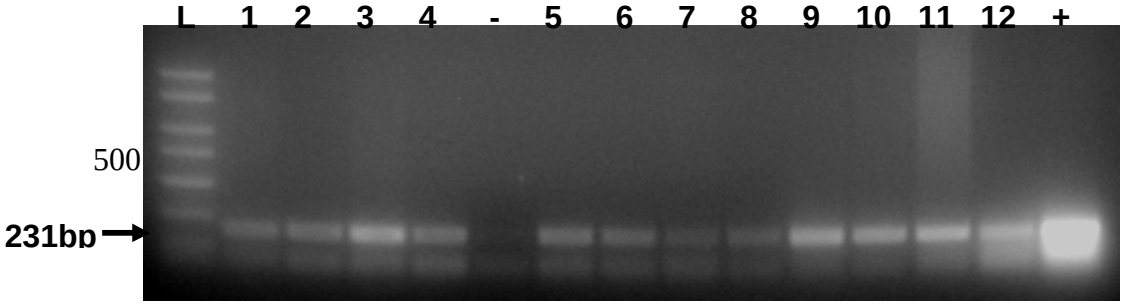


Figure 3.3 Agarose gel electrophoresis of RT-PCR amplified products (231bp CBSV coat protein gene fragment) for samples from CBSV transmission experiments using CBSV-specific primer pair CBSV 10F and CBSV 11R. RNA was isolated from 0.1 g of leaf tissue from collected samples. Lanes L-1kb plus molecular weight marker (Invitrogen); 1-2, samples with buffer A; 3-4, samples with buffer B; -, negative control from tissue culture plantlet; 5-7, samples with cutting tools for cvs. Albert, Mreteta and Supa; 8, sample with leaf harvesting; 9-10, graft samples with CBSV infected scion; 11-12, graft samples with CBSV infected root stock; +, Positive control from CBSV infected plant.

3. 5 DISCUSSION

In sap transmission, higher transmission rates of CBSV and earlier expression of CBSD symptoms was achieved with buffer B (54 %) compared to buffer A (29 %). Based on the composition of the two buffers, buffer A had bentonite and glycine added but the pH was adjusted to pH 9.0 unlike the pH 7.5 of buffer B. Bentonite was reported to improve the infectivity of the potato spindle tuber viroid (Grasmick and Slack, 1987). However, the role of glycine in enhancing virus infectivity seems limited apart from being a conjugate inhibitor of the B6 protein (Anon, 1999). With bentonite as a component of the buffer, greater efficiency was expected from buffer A compared to buffer B, but the reverse was realized. The difference in molar concentration between the two buffers (0.05M for buffer A and 0.1M for buffer B) possibly contributed to the variation in infectivity. The higher molarity in buffer B probably increased its efficiency compared to buffer A. The buffer pH may also affect the infectivity efficiency. Since buffer B had its pH adjusted to 7.5 unlike pH 9.0 of the buffer A, that factor could be vital to the infectivity of CBSV.

Graft inoculation was the most efficient and effective of the techniques assessed because 100 % transmission was attained. The relatively shorter time required for the virus detection and symptoms expression when the CBSV-free scion was grafted onto an infected root stock suggests this technique to be the best for transmission studies. Earlier detection of CBSV by this grafting technique suggests that CBSV titre was higher in the lower parts of the plants particularly the roots compared to the shoots. This is consistent with the classical study on the nature of virus movement in plants as demonstrated by Samuel, (1934) with *Tobacco mosaic virus* (TMV). He demonstrated that after infection and replication, TMV particles were

systemically translocated through phloem to lower parts of the tomato plant and re-distributed to the youngest leaves and the rest of plant shoots. Unlike in other forms of CBSV inoculation that were tested, the expressed symptoms were clear and typical of CBSD. The findings in this study are consistent with the CABRI (1998) report, that grafting is an effective way of transmitting virus strains that are not readily or not at all mechanically transmissible to susceptible host plants.

CBSV was not transmitted through seeds which supports earlier findings (Maruthi *et al.*, 2005). The lack of CBSV transmission through seeds derived from infected mother plants suggest that the morphology of the reproductive organs of cassava does not allow entrance and survival of CBSV in the embryo. According to Carroll (1972), non-seed transmitted viruses are not detected in pollen or embryos. In contrast, there could be some inhibitors of virus infection of seeds as reported by Gunnery and Datta (1987). In their studies, a low molecular weight RNA was isolated from barley embryos that specifically inhibited the initiation of protein synthesis. Such a molecule was suggested to play a role in inhibiting virus synthesis and seed transmission in some species. The lack of transmission of the virus through seeds suggests a safe base for cassava breeding work. It offers a great opportunity for conventional breeding for resistance to CBSD, through selective crossing. The ease with which breeder seeds may be exchanged regionally and internationally without fear of transmitting CBSV is also an added benefit of the information gained through this study.

The inability of CBSV to be spread from infected root debris is important in establishing that cassava plants are not at risk from the remains of the previous crop. Virus-free cuttings of

cassava can therefore be planted in a field previously grown with CBSV-infected material with no apparent risk of carry-over of virus inoculum. The inability of the cuttings to sprout after pre-exposure to infected debris suggests the likely presence of inhibitive chemicals that acted allelopathetically on the cuttings. The lack of any sign of fungal infections on the pre-soaked cuttings discounted the possibility of fungi being a cause of cutting deaths. However, detailed studies are required to confirm this assumption.

The infection rates of 22 % for cutting tools and 7 % for leaf harvesting suggested that farming agronomic practices can make an appreciable contribution to the spread of CBSV. For the first time, it was demonstrated that CBSV ca be transmitted through normal agronomic operations. CBSV transmission to only one plant through leaf harvesting was too low to warrant scientific conclusion. However, given the small number of the test plants the contribution of leaf harvesting could be very substantial. Further studies with many more test plants would be needed to justify the contribution of leave harvesting to CBSV spread. Although the transmission efficiency was relatively low, the cumulative contribution could be significant because of the multiplication rate of cassava in which each cutting can produce an average of ten cuttings per annum (Otoo, 1996). The observed transmission of CBSV through contaminated tools is not uncommon among viruses. Contaminated tools have been reported to transmit spindle tuber viroid and Potato virus X in potato (Manzer and Merriam, 1961) as well as citrus exocortis viroid in citrus (Broadbent *et al.*, 1968).

The current study demonstrated that sap transmission of CBSV is achievable when the inoculum is obtained from the youngest symptomatic leaves of infected plants with buffer pH

adjusted to 7.5. New transmission of the virus is neither expected from seeds nor infected plant debris. This may allow for the possibility of continuous cropping, especially in areas where the area of productive land is limiting. Graft transmission of the virus is the best technique for indexing and detection of the virus in any infected stock of CBSV-susceptible plants. Best and quickest results are achieved when susceptible virus-free scions are grafted onto infected rootstocks. Cutting tools, such as knives, used in preparation of cassava cuttings and harvesting of cassava leaves for use as vegetable may contribute to the spread of CBSD.

CHAPTER 4

VARIABILITY OF CASSAVA BROWN STREAK DISEASE SYMPTOMS AND THE RELATIONSHIP BETWEEN VIRUS INFECTION AND SYMPTOM EXPRESSION IN ON-FARM CASSAVA

Manuscript in preparation for submission to Plant Pathology

4.1 SUMMARY

Diagnosis of cassava brown streak disease (CBSD) has for long been based on foliage and root symptoms expression by infected plants. Variability in patterns of symptom expression between varieties and seasons however, has meant that symptom-based diagnosis is unreliable. The current study identified three major types of foliage and root symptoms associated with CBSD. The newly described CBSD symptoms includes; spotty foliage chlorosis, brown necrotic internal tissue at the base of the leaf petiole and chalky root necrosis. The relationship between symptom expression and the presence of *Cassava brown streak virus* (CBSV) through RT-PCR was also established. Plant parts most suitable for detection of CBSV by RT-PCR were identified. Foliage CBSD symptoms could be unreliable indication of CBSV infection. Moreover, CBSV may not be uniformly distributed within the infected plants.

67 % of tested samples were from plants that were both symptomatic and shown to be infected by CBSV. 22 % were free from CBSV although apparently symptomatic and 7 % were CBSV-infected but symptomless. CBSV was detected in flowers, fruits, apical buds, young tender leaves, newly-opened leaves, youngest symptomatic leaves, and the tender top green portion of the stem and non-necrotic storage root tissues. Virus-free plants could not be

regenerated from CBSD-affected plants of cvs. AR 49/2, Cheupe and Nachinyaya except in cv. Albert, where three of the five symptomless plants were confirmed to be CBSV-free by RT-PCR.

4. 2 INTRODUCTION

Diagnosis of CBSD has always been dependent on symptom expression on cassava (Hillocks, 1997; Hillocks and Thresh, 1998; Hillocks *et al.,* 1999; Legg and Raya, 1998) in the absence of a reliable method of detecting the causal virus (CBSV). The importance of a thorough knowledge on disease symptoms for effective diagnosis has been documented (Agrios, 1997). In virology, disease symptoms are particularly important because most viral diseases are named after the nature of symptoms caused (Matthews, 2002). The reported symptoms typical of CBSD include vein chlorosis and/or chlorotic blotches in mature leaves which occur without distortion of the lamina, brownish-black streaks on the stem and brownish-black necrosis in the root cortex. Detailed descriptions of CBSD symptoms were given by Nichols (1950a) and reviewed later by Hillocks and Jennings (2003).

Recently, it was observed increasingly that some of the cultivars being grown by farmers do not show CBSD symptoms despite being infected by the virus (G. Rwegasira, unpublished data). In cultivars where CBSV-infection is not apparent from symptoms, false conclusions could be made considering the plant to be CBSV-free although it may be infected. Nichols (1950a) reported similar confusion in CBSD symptom expression. In the current study, new molecular diagnostic techniques were used to gain an improved understanding of the relationship between CBSV infection and symptom expression.

The studies reported here aimed at exploring the diversity of CBSD symptoms and the relationship between CBSV infection and symptoms expression. Specific objectives of the studies were:

(i) To define the major types of symptoms,

(ii) To determine the reliability of symptoms in diagnosing infection,

(iii) To identify the plant parts which are most suitable for CBSV detection,

(iv) To determine the possibility of regenerating CBSD-free plants from cuttings collected from diseased plants.

4. 3 MATERIALS AND METHODS

4. 3. 1 Major types of CBSD symptoms

A diagnostic survey was conducted in 91 farmers' cassava fields in the major cassava-growing Zones of Tanzania, from August to October 2005, to document the symptoms associated with CBSD. The surveyed Zones included; 'Coastal' (coastline stretch along the Indian Ocean from Tanga Region in the north-east to Mtwara in the south-east), 'Lake' (along Lake Victoria basin in the north-western part of Tanzania, extending to the northern shore of Lake Tanganyika) and 'Southern' (south to south-west from the Mozambique border to the shores of Lake Malawi and the southern shore of Lake Tanganyika). The survey involved traversing along passable roads, and stopping at regular intervals of 100 km where cassava fields were found. The major types and nature of symptoms expressed by CBSD-affected plants were documented. Whenever two types of symptoms occurred together the dominant one was considered. Written descriptions of symptom characteristics were made and digital images taken.

One to three cuttings were obtained from each of the CBSD-symptomatic plants sampled and maintained in pots at MARI for close monitoring of the expressed symptoms.

4. 3. 2 Field and screen-house experiments on CBSD symptoms

Representative cassava cultivars with the major types of foliage and root symptoms noted during the survey were identified, and grown in the field and in pots at SRI, Kibaha. The six selected cultivars included five from farmers' fields (Albert, Cheupe, Kibaha, Nachinyaya and Namikonga) and one from experimental fields at SRI, (AR 49/2). Characteristic CBSD symptoms were established for each of the six cultivars (Table 4.1) prior to experimentation.

Table 4.1 Cultivars with diverse CBSD symptoms selected for symptomatology experiment

Characteristic symptoms	**Experimental cultivar**
1. Foliage vein chlorosis. Necrosis absent in roots for more than 9 months after planting.	Nachinyaya
2. Early foliage chlorotic blotches at less than 2 months. Brown mass root necrosis at 6 months.	Albert, Cheupe
3. Early foliage chlorotic blotches at 2 months. Late root symptoms, after 9 months. Random blackish/brown necrotic mass in tuberous roots.	Kibaha
4. Early foliage vein chlorosis. Rapid die-back. Early root symptoms at 6 months. External constriction and fissures. Necrotic specks in the root cortex.	AR 49-2
5. Rare but spotty foliage symptoms. Root symptoms uncommon. Chalky necrosis.	Namikonga

In the field experiment, thirty-six plants per plot for each of the six cultivars were planted and replicated three times giving a total of 108 plants per cultivar per season. The experiment was conducted for two consecutive years, 2006/2007 and 2007/2008. The nature and gradual changes of the different types of shoot symptoms were recorded monthly as the plants grew. Root symptom assessment was done monthly beginning six months after planting by piece-meal harvesting of roots (one root per plant) from any three randomly selected plants for each symptom type until at 12 months when harvesting was done. A similar pot experiment was conducted in the screenhouse for closer examination of the daily changes in CBSD shoot symptoms.

4. 3. 3 Relationship between symptoms expression and detection of CBSV

During the survey, one leaf sample was selectively collected from one CBSD symptomatic plant in each of 91 fields. However, in the absence of any symptomatic plants, a sample was chosen from a randomly selected plant. Test samples were collected using gloves and placed individually in self-sealable plastic bags and kept in a cool box after which, they were sent to the laboratory at MARI for CBSV detection. The CBSD-related symptoms observed on each plant sampled were recorded. The guanidium thiocyanate method was used to isolate total RNA from the collected samples. Using CBSV coat protein (CP) gene-specific primers (CBSV10/11) (Monger *et al.*, 2001a), triplicate RT-PCR was run at optimum conditions and the virus-infected samples were delineated. The RT-PCR results were compared to the field CBSD symptoms recorded from the samples.

4. 3. 4 Isolation of RNA

Total RNA was extracted from 0.1g fresh leaf tissue in 4 M guanidium thiocyanate (Sigma, 59980) buffer mixed with 2-mercaptoethanol (Sigma, M3148) at a ratio of 1:125 (w:v) of 2-mercaptoethanol to guanidium by using a sterile mortar and pestle. Five hundred µl of the lysate were transferred to a 1.5 ml eppendorf tube, 500 µl of 2.0 M sodium acetate was added and the tube contents were thoroughly mixed. Four hundred µl of 24:1 [chloroform ($CHCl_3$) to isoamyl alcohol (C_5H_2O)] were added to the tube and the contents were mixed and incubated on ice for 10 minutes. Tubes were centrifuged at 13000 rpm for 15 minutes and 450 µl of supernatant transferred to new tubes. Five hundred µl of ice-cold isopropanol were added to precipitate the RNA and the tube was then incubated at –20 ^{0}C for 10 minutes. The chilled contents were centrifuged at 13000 rpm for 10 minutes and the supernatant discarded. The RNA pellet was air-dried after washing with 500 µl of 75 % ethanol and centrifuging at 13000 rpm for 2 minutes. Thirty five µl of RNase free water were added to dissolve the RNA pellet for analysis in RT-PCR.

4. 3. 5 Nucleic Acid Amplification by RT-PCR.

Triplicate RT-PCR was performed in a one-step reaction using superscript ^{TM} III RT/Platinum® *Taq* Mix System (Invitrogen Life Technologies) using a GeneAmp PCR system 9700 (Applied Biosystems, UK). The primers, CBSV 10F and 11R (Monger *et al.,* 2001a) designed to amplify a 231 bp segment of the CP gene were used. Each 50 µl reaction mixture comprised 25 µl of 2X reaction mix (buffer with 0.4 mM of each of dNTP, 3.2 mM $MgSO_4$ and stabilizers), 1 µl of RNA, 0.4 µl of each of the forward and reverse primers, 1 µl of superscript RT-Taq (Invitrogen 12574-026) and 22.2 µl of sterile water. The PCR conditions were as

follows; Initial cDNA synthesis and denaturation; at 55°C for 0.5 min. and 94°C for 1 min, denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min (in 35 cycles) followed by final extension at 72°C for 10 min.

4. 3. 6 Analysis of RT-PCR product.

RT-PCR products were separated electrophoretically in 1.2 % agarose gels in 0.5X Tris Acetate EDTA (TAE) buffer, for 1 hour at 92 volts. Amplicons were visualised by staining with ethidium bromide (0.01 µl/ml) under ultraviolet (UV) light and recorded using an image analyser (Syngene).

4. 3. 7 Plant parts suitable for CBSV detection

An experiment to determine the most suitable plant tissues for CBSV detection was conducted at MARI. Tissues from various parts of four diseased cassava cultivars; Albert, Cheupe, Kibaha and Nachinyaya were collected from the field-grown plants and tested for CBSV. Test samples included seeds, fruits, flowers, apical leaf buds, tender young leaves, young fully-expanded leaves, mature symptomatic leaves and old near-senescent leaves. The stem samples included peelings from the young green tender portion, the middle section of the stem and the mature woody stem. Root samples were: the total necrotic tissue, the thin layer of soft tissue at the necrotic margin and non-necrotic tissue of the roots outside the necrotic margin. Triplicate samples were collected from five CBSD-affected plants for each of the four cultivars. The experiment set up was as follows: One sample for each of plant tissues x 5 plants for each cultivar x 4 cultivars. For each tissue type, 20 samples were tested. Considering the 14 different tissues types sampled, the number of tested samples was 14 tissue types x 5 plants x 4 cultivars

giving a total of 280 different samples. One CBSV-free leaf sample for each cultivar was included as a negative control during testing. CBSD characteristic symptoms on each of collected samples were recorded. CBSV was detected with RT-PCR using the CP gene-specific primers.

4. 3. 7 Regeneration of healthy plants from diseased cuttings

This experiment was based on the observation in previous experiments that cuttings from seemingly CBSD-affected plants tended not to exhibit CBSD symptoms when replanted at the same or different locations (G. Rwegasira, unpublished data). A pot experiment to determine whether CBSV-free plants may be obtained from diseased mother plants, expressing CBSD symptoms of varying severity was conducted in pots in the screenhouse at MARI. All planting materials were collected from CBSD symptomatic plants (severity levels 2 through 4), pre-confirmed for CBSV-infection by RT-PCR. Different sections of stem were used as sources of planting material. The cultivars: Albert, AR 49/2, Cheupe and Nachinyaya were tested. Stems about 2.5 m in length were cut into three major sections. The mature bottom portion (up to 0.5 m above ground, highly lignified and with short internodes), the middle semi-mature portion (0.6 m - 1.0 m above ground and normal internode length) and the immature upper portion (above 1.0 m, mostly green, more or less succulent and with irregular internode length). Five cuttings were obtained from each of the three sections in each cultivar. A total of 45 plants were tested for each cultivar. The whole experiment comprised 180 potted plants. CBSD symptoms were recorded monthly for three consecutive months beginning at 2 months after planting. Detection of CBSV was done in leaf tissues collected from symptomless plants.

4. 4 RESULTS

4. 4. 1 Types of CBSD field symptom

Same plants were assessed for CBSD foliage (91 plants) and root (81 plants) symptoms. CBSD foliage symptoms were observed in 81 of the 91 plants assessed for leaf symptoms and root symptoms were recorded in 47 of the 81 plants assessed for root symptoms. Several major types of leaf and root symptoms were recorded (Table 4.2). Chlorotic blotch was the major foliage symptom followed by spots and vein chlorosis. In some instances, the chlorotic blotches appeared together with vein chlorosis although such occurrence was rare. Brown mass necrosis and chalky necrosis were the most prevalent symptoms diagnosed in roots of CBSV-infected plants, with greater than 34 % and 19 % incidence, respectively.

Table 4.2 CBSD leaf and root symptoms recorded during survey

Types of CBSD leaf symptoms (N = 91)				Types of CBSD root symptoms (N=81)			
Symptomless leaf	Chlorotic blotches	Chlorotic spots	Veinal chlorosis	Brown/black necrosis	Chalky necrosis	Necrotic specks	Symptomless
10 (11%)	42 (46.1%)	28 (30.8)	11 (12.1%)	28 (34.6%)	16 (19.7%)	3 (3.7%)	34 (42%)

Proportionate distribution of the major CBSD symptoms recorded on sampled plants suspected to be CBSV-infected during the survey. Same plants were assessed for root and foliage CBSD symptoms.

4. 4. 2 Relationship between foliage CBSD symptoms and CBSV detection

CBSV detection by RT-PCR indicated the presence of the virus in both symptomatic and symptomless samples (Figure 4.1). 61 of 91 (67 %) of the tested leaf samples were both symptomatic and infected with CBSV. 20 of 91 (22 %) leaf samples did not give an RT-PCR amplicon for CBSV despite being symptomatic for CBSD and 6 of 91 (7 %) leaf samples were CBSV-infected but symptomless. Only 4 of 91 (4 %) tested leaf samples were symptomless and CBSV-negative.

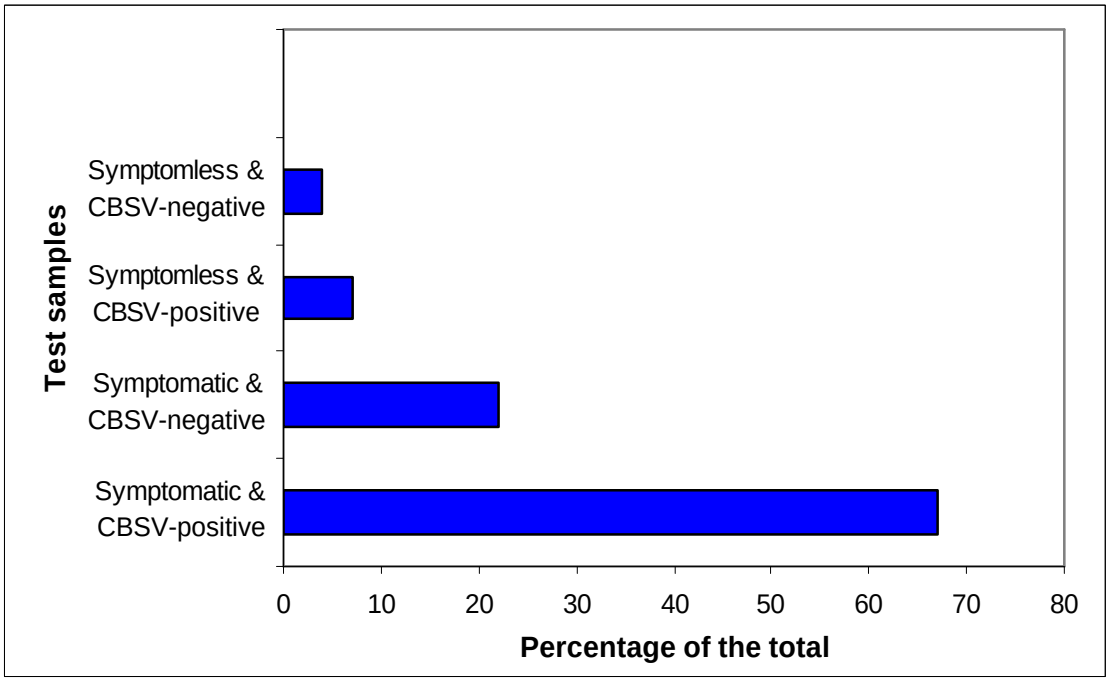


Figure 4.1 Relationship between CBSV-infection and presence of leaf symptoms on 91 tested samples collected during the survey.

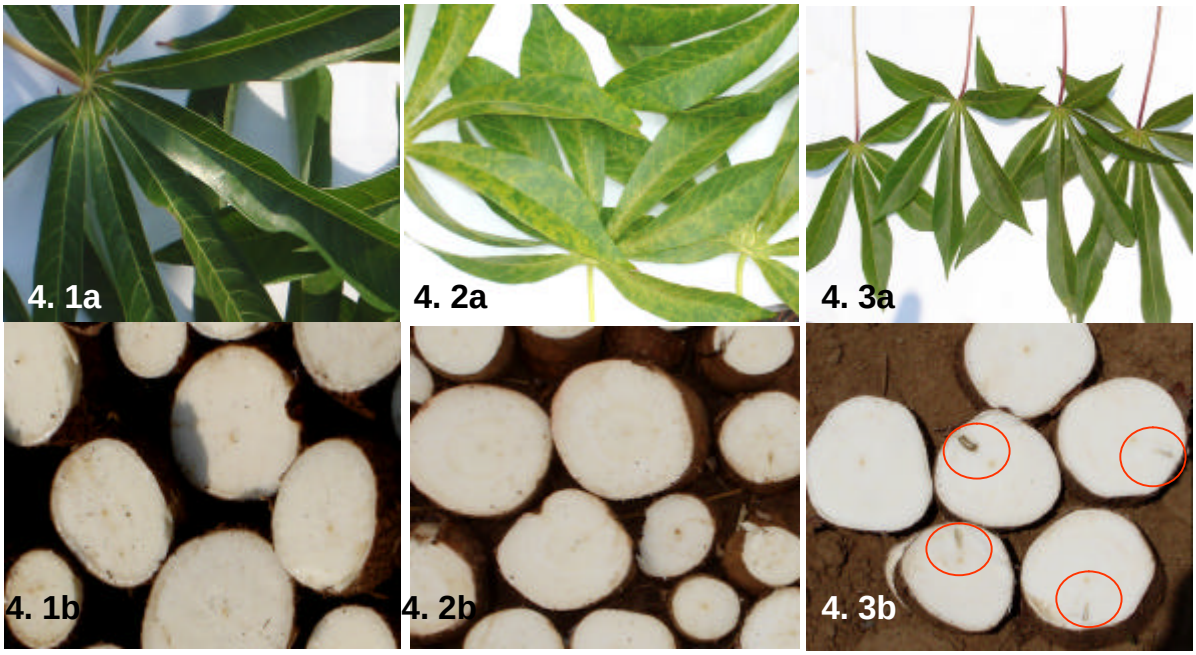


Plate 4.1a - 4.3b Indication that CBSD symptoms may only be suggestive of CBSV infections. **4.1a & b**; CBSV-infected, **4.2a & b**; CBSV-free; **4.3a & b**; CBSV-infected.

Some plants without leaf and root symptoms were shown to be CBSV-infected (Plate 4.1a & b), a few plants had CBSD-like foliage symptoms but no root symptoms and were CBSV-free (Plate 4.2a & b). Some CBSV-infected plants did not exhibit any foliage symptoms but had root necrosis (Plate 4.3 a & b)

4. 4. 3 Foliage symptoms of CBSD and CBSV detection in surveyed Zones

The relationship between the three major types of foliage CBSD symptoms and presence of the virus was established for the 81 samples that exhibited foliage symptoms. The Coastal Zone had most of CBSV-infected samples with chlorotic blotches 13/33 (39 %), followed by the Lake 10/33 (30 %) and Southern Zones 10/33 (30 %) (Table 4.3a). Most CBSV-positive leaf samples in the Lake Zone had chlorotic spots 8/20 (40 %) and veinal chlorosis, 5/8 (63 %). The Southern Zone had the least number of samples in any of the three symptom types.

Table 4.3a Relationship between the mean incidence of the three major types of foliage CBSD symptoms and CBSV detection by RT-PCR in the three surveyed zones

Zones	CBSD mean foliage incidence, symptom type and CBSV detection by RT-PCR (N = 81)					
	Chlorotic blotches (n = 43)		Chlorotic spots (n = 28)		Veinal chlorosis (n = 10)	
	CBSV Positive	CBSV Negative	CBSV Positive	CBSV Negative	CBSV Positive	CBSV Negative
Lake Zone (35)	10 (12.3 %)	7 (8.6 %)	8 (9.2 %)	5 (6.1 %)	5 (6.2 %)	0 (0 %)
Coastal Zone (26)	13 (16.1 %)	1 (1.2 %)	7 (8.6 %)	2 (2.8 %)	2 (2.8 %)	1 (1.2 %)
Southern Zone (20)	10 (12.3 %)	2 (2.5 %)	5 (6.6 %)	1 (1.2 %)	1 (1.2 %)	1 (1.2 %)
TOTAL (81)	33 (40.7 %)	10 (12.3 %)	20 (24.7 %)	8 (9.9 %)	8 (9.9%)	2 (2.5 %)

Numbers in brackets are percentage of the total number of the 81 samples with foliage symptoms

The general trend (Table 4.3b) indicated that 33/81 (41 %) samples were CBSV-positive and had foliage chlorotic blotches; 20/81 (25 %) samples were CBSV-positive and had foliage chlorotic spots; whereas 8/81 (10 %) samples were CBSV-positive and had foliage veinal chlorosis. The Coastal Zone had the highest number of CBSV-infected samples 22/26 (85 %) regardless of the type of symptoms, followed by the Southern zone 16/20 (80 %). The Lake zone had the least infected samples 23/35 (66 %). Most plants 8 of 10 (80 %) with apparent veinal chlorosis were CBSV-infected.

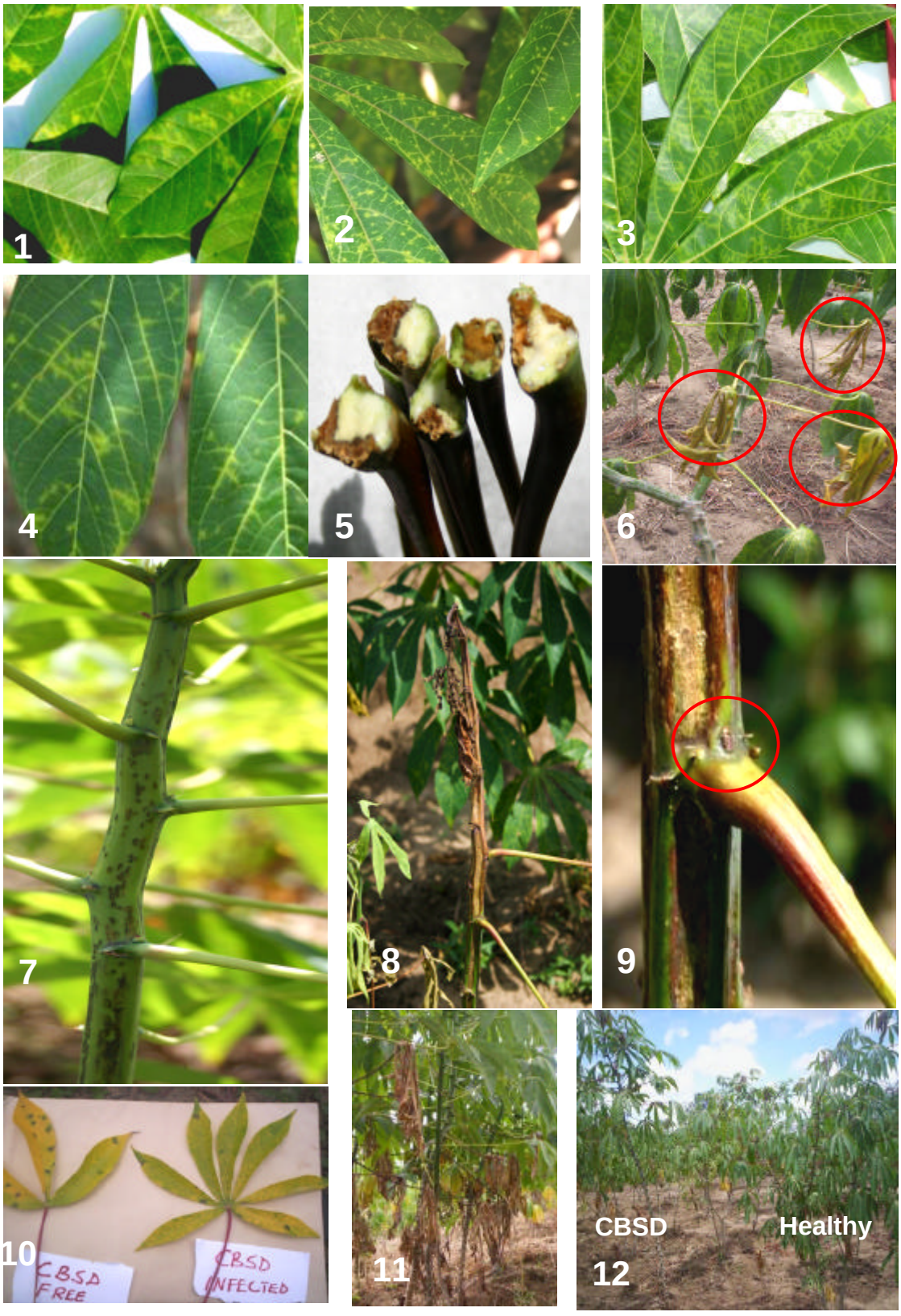
Table 4.3b Relationship between each of the three types of foliage CBSD symptoms and number of CBSV-infected plants as per detection by RT-PCR

ZONES	Chlorotic blotches (n=43) CBSV-positive/n	Chlorotic spots (n=28) CBSV-positive/n	Veinal chlorosis (n=10) CBSV-positive/n	TOTAL	Total CBSV-positive
Lake Zone	10/17	8/13	5/5	23/35	66 %
Coastal Zone	13/14	7/9	2/3	22/26	85 %
Southern Zone	10/12	5/6	1/2	16/20	80 %
TOTAL	33/43	20/28	8/10	61/81	
% CBSV-positive	77 %	71 %	80 %	75 %	

4. 4. 4 Nature of foliage symptoms of CBSD

Chlorotic blotches were observed in 33/61 (54 %) of CBSV-infected samples with foliage symptoms. Blotches appeared to have started at various points across the leaf area and later coalesced presenting a mosaic-like appearance. Careful monitoring of this type of symptom in the field and in screenhouse experiments showed that the brownish chlorotic blotches with irregular-shaped margins first appear in the leaf lamina and later expand to cover most of the tertiary and secondary vein area leading to larger blotches (Plate 4.1). Leaves affected in this way were not malformed.

Chlorotic spots accounted for 20/61 (33 %) of symptoms associated with CBSV infection in the survey sample. These were circular chlorotic patches with more or less smooth margins (Plate 4.2).



Plates 4.1-12 Foliage and stem symptoms of CBSD on diseased plants:

Plates 1– 6: Foliage symptoms of CBSD: 1- chlorotic blotch, 2- chlorotic spots, 3- vein chlorosis, 4– complex of blotchy and vein chlorosis, 5- necrosis in leaf petiole bases, and 6- non-uniform senescence of leaf petiole. **Plates 7– 9:** Stem symptoms of CBSD: 7- blackish necrotic spots on stem of CBSD-affected plant, 8 – stem die-back due to CBSD, and 9 – death of auxiliary buds on the stem during die-back. **Plates 10– 12:** Senescence symptoms: 10 – comparison between senescent leaf of CBSD affected and healthy plant, 11 – failure of senesced bottom leaf petiole to detach, and 12 – poor leaf retention

Observations in the field and screenhouse experiments indicated that CBSD chlorotic spots start in the leaf lamina expanding to cover the tertiary and secondary veins and later coalesce to form a blotch-like appearance. CBSD chlorotic spots are often difficult to differentiate from chlorotic blotches because at an advanced stage the two types of symptom tend to look alike. However, unlike the chlorotic spots, blotches expand through veins to assume the angular and irregular margins. Like other types of symptoms, the leaf shape and other normal morphological characteristics were retained.

Vein chlorosis was evident in 8/61 (13 %) of CBSV-positive samples collected during survey. Experimental observation showed that vein chlorosis starts in the tertiary veins forming a net-like appearance which expands to affect the secondary veins (Plate 4.3). Finally the net-like chlorosis affects the primary veins and assumes a feathery mosaic appearance. Vein chlorosis is occasionally associated with chlorotic blotches (Plate 4.4).

4. 4. 5 Leaf petiole symptoms

An important symptom recorded in a few cultivars was the presence of necrotic internal tissue at the base of the leaf petiole when detached from the stem. The necrotic tissue appeared as brown masses of necrotic inclusions in tissues at the petiole base, interfering with the abscission layer of the nearly senescent leaves (Plate 4.5). The brown mass was mainly comprised of dead tissues. Experimental observations on this type of symptom showed that the necrosis starts to develop in the leaf petiole as it approaches maturity. The dark-brown necrotic mass in the petiole base expands as the leaf matures until senescence. The whole portion that links the petiole to the stem may be affected. Leaves with necrotic masses in the petiole shrink and dry up quickly, do not naturally detach from the stem even after senescence and often remain attached to the stem and inclined downwards.

Irregular senescence in mature leaves was also observed during the dry season in some of the CBSD-affected cvs. These included: Albert, AR 49/2 and Cheupe. The younger leaves senesced earlier than the older leaves of the same plant (Plate 4.6). In leaf petioles of CBSD symptomatic leaves, tissue death started at the base of the leaf lamina extending to cover the whole leaf lobes and subsequently to the ligament at the node on the stem. Senesced CBSD-symptomatic leaves had a chlorotic mosaic-like appearance that was quite distinct from that of CBSD-free plants (Plate 4.10). Unlike the diseased plants, senescence in CBSD-free plants started on the oldest bottom leaves ascending gradually with maturity and the leaf petioles were uniformly yellow. During the dry season, senescent leaf petioles of the CBSD-affected plants bent at a sharp angle and dried up but did not detach from the stem (Plate 4.11). This characteristic was observed repeatedly in cvs. AR 49/2, Cheupe and Kibaha.

Poor leaf retention was another characteristic of CBSD-affected plants. This was observed in cvs. Albert and Kibaha. The comparison between ten CBSV-free and CBSD-affected plants (Plate 4.12) grown for two consecutive seasons (2006/2007 and 2007/2008) (data not shown) indicated that CBSD-free plants retain more leaves in all seasons compared to the diseased ones. Lack of CBSV-free cuttings prevented a parallel comparison in other varieties. Since only two varieties were tested, the leaf retention phenomenon could be cultivar-specific. Another experiment will be required to justify this observation.

4. 4. 6 Stem symptoms

A mixture of brownish-black necrotic spots, slit-like lesions and streaks on green stem portions were the major symptoms observed in CBSD-affected plants. Striping of the stem epidermis in some plants with apparent necrotic lesions on the surface, revealed a deeper development of the lesion into the cortical portion but not penetrating the pith. The expression of stem symptoms was variety-dependent and only a few sensitive cultivars were affected.

In experimental plants, the stem symptoms started as single small blackish necrotic spots. Spots increased in number with time on the green part of the stem (Plate 4.7). The necrotic spots coalesced into slit-like lesions which later produced a blackish-brown streak. In severe cases, the tender growing parts of affected plants started withering and leaves subsequently dried up. Affected stems started dying from the tip downwards, the phenomenon described as ‘die back’ (Plate 4.8). In sensitive cultivars like AR49/2, necrotic spots on the stem were observed as early as 65 days after planting and the time taken from first appearance of stem necrotic spots to die-back ranged between two to three weeks. In the process of die-back, all the axillary buds were

killed (Plate 4.9) and ultimately the whole plant died. The death of tissue was faster in the internode compared to the nodal section of the stem. Apart from the cv. AR49/2, in which stem lesion development often culminated in die-back, none of the test cultivars exhibited die-back. Frequent stem lesions recorded in Albert, Cheupe and Kibaha at certain periods of the year never led to die-back.

4. 4. 7 Root symptoms

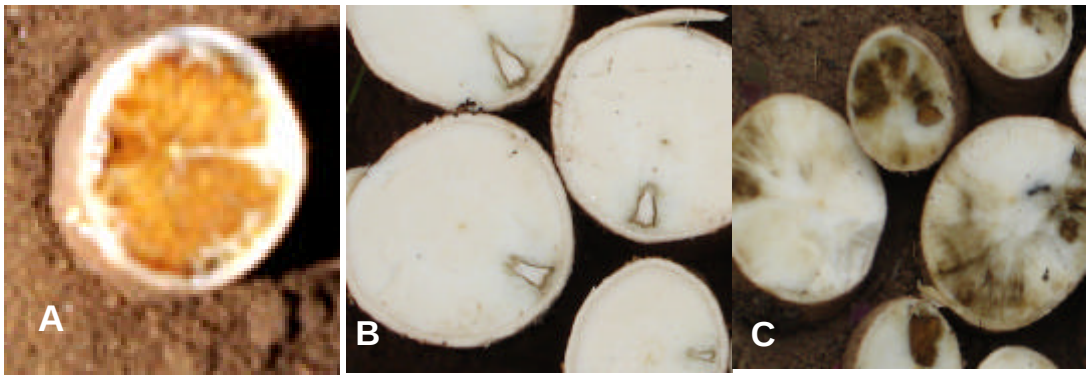
The external root symptoms observed during the survey were irregular constrictions associated with localised fissures. This observation was limited to only 2 of the 47 cultivars with root symptoms (data not shown). Internal root necrosis was the most destructive symptom recorded during the survey. It was characterised by the yellow or brownish-black inclusions of dead tissue in the cortical portion of the storage roots. The presence of root necrosis was associated with foliage symptoms in 43 of 47 (91 %) symptomatic plants (Table 4.4).

Brown mass necrosis was the most common symptom which accounted for more than 59 % of survey samples with root necrosis. It comprised irregular-shaped, yellowish to brownish-black necrotic masses of varying size present in the root cortex. Observations in experimental plants revealed that this type of symptom started as randomly distributed necrosis in the root cortex. The individual necrotic flecks enlarged as the root grew and merged into one or more large irregular-shaped masses. In some instances, necrosis expanded to cover the whole storage root (Plate 4A). The colour of the necrotic masses varied from yellowish to dark brown. Considering the proportion of root volume affected, this was the most damaging symptom type. This type of symptom was most commonly observed in the Coastal Zone.

Table 4.4 Relationship between the mean incidence of the three major types of root CBSD symptoms and CBSV detection by RT-PCR in the three surveyed zones

	Mean CBSD root incidence, symptom type and CBSV detection by RT-PCR								
	Brown mass necrosis (n = 28)			Chalky necrosis (n = 16)			Necrotic specks (n = 3)		
Surveyed Zones	CBSV Positive	CBSV Negative	Total	CBSV Positive	CBSV Negative	Total	CBSV Positive	CBSV Negative	Total
Lake Zone	5 (10.6 %)	0 (0 %)	5	12 (25.5 %)	0 (0 %)	12	0 (0 %)	0 (0 %)	0
Coastal Zone	15 (31.9 %)	0 (0 %)	15	1 (2.1 %)	0 (0 %)	1	2 (4.3 %)	0 (0 %)	2
Southern Zone	8 (17 %)	0 (0 %)	8	3 (6.4 %)	0 (0 %)	3	1 (2.1 %)	0 (0 %)	1
TOTAL (47)	28/47 (59.6 %)	0/47	28	16/47 (34 %)	0/47	16	3/47 (6.4 %)	0 (0 %)	3

CBSV detection on root samples was done by RT-PCR using samples excised from the white (non-necrotic) portions of the roots. All plants with CBSD root symptoms were found infected by CBSV



Plates 4A-C, Major root symptoms of CBSD. **A**– Brown mass necrosis, **B** – Chalky necrosis and **C** –Necrotic specks.

In some cultivars, this type of symptom appeared as random necrotic lesions comprised of brownish-black spots in the root cortex. At an advanced stage the necrotic spots coalesced to affect the whole storage tissue. The root texture was softer in the necrotic portion compared to the non-necrotic parts. Most roots that exhibited this type of symptoms had started to develop soft rots.

Chalky necrosis was the second important root symptom that accounted for 33 % of root necrosis in the plants assessed during the survey. In this type, necrosis in a small portion of the root tissues assumed a triangular shape, with a chalky-white mass bordered by the brownish-black margin (Plate 4B). Slight textural differences existed between the necrotic and the rest of the root tissue. The chalky-white necrotic portion had a drier and harder texture compared to the unaffected root cortex. In experimental plants, it was established that this type of necrosis was localised at a few points along the length of the root. It rarely expanded to cover much of the cortical portion of the roots. Plants with this type of symptom rarely showed foliage symptoms.

Necrotic specks appeared like thin, hard burnt brick impregnated within the root cortex (Plate 4C) and were observed in *ca* 6 % of survey samples of those with root symptoms. The necrosis had a coarse and hard texture, contrasting with the rest of the cortical tissue. In experimental plants, necrotic specks grew occasionally along the longitudinal section of the roots forming discrete entities. The margin of necrotic flecks was tough with clear demarcation from the non-necrotic cortical root tissue. In some instances, specks were seen only on cross-sectioning the affected root at several points at short intervals. Unlike the brown mass necrosis, the diameter of necrotic specks rarely exceeded 10 mm regardless of the root size.

Table 4.5 Relationship between CBSD symptoms on different parts of the sampled plants and CBSV-infections on test samples as per detection by RT-PCR

Plant parts with CBSD symptoms	Zones				
	Number of CBSV-positive samples			CBSV-free with CBSD symptoms	Total*
	Lake zone	Coastal zone	Southern zone		
Leaf symptoms	23/81 (28.4 %)	22/81 (27.16 %)	16/81 (19.75 %)	20/81 (24.69 %)	81
Stem symptoms	11/57 (19.3 %)	24/57 (42.11 %)	17/57 (29.82 %)	5/57 (8.77 %)	57
Root symptoms	17/47 (36.17 %)	18/47 (38.3 %)	12/47 (25.53 %)	0/47 (0 %)	47
Leaf and stem	11/48 (22.92 %)	22/48 (45.83 %)	15/48 (31.25 %)	0/48 (0 %)	48
Leaf and roots	15/40 (37.5 %)	18/40 (45 %)	7/40 (17.5 %)	0/40 (0 %)	40
Stem and roots	14/40 (35 %)	17/40 (42.5 %)	9/40 (22.5 %)	0/40 (0 %)	40
Leaf, stem and roots	10/36 (27.8 %)	17/36 (47.2 %)	9/36 (25 %)	0/36 (0 %)	36

* indicates the total number of symptomatic plants in the respective category

The comparative analysis of CBSD symptoms in different parts of the assessed plants, from which foliage samples for CBSV detection was collected (Table 4.5) indicated that foliage symptom was most prevalent compared to the stem and root symptoms. CBSV was detected in all plants with root symptoms. The data suggest that plants with CBSD symptoms on more than one part contain detectable CBSV compared to when symptoms are exhibited on one part. All plants with a combination of leaf and stem, leaf and roots, stem and roots, and a combination of leaf, stem and root symptoms had detectable CBSV. Thus, CBSD diagnosis by using observable symptoms should always consider the presence of symptoms on more than one parts of the plant.

4. 4. 8 Detection of CBSV in different plant parts

Triplicate RT-PCR tests done on different plant parts suggested considerable variation in detectable CBSV in different plant tissues (Figure 4.2).

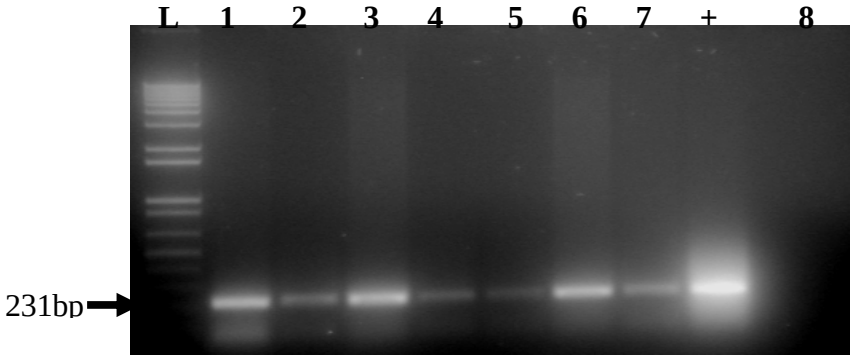


Figure 4.2 Agarose gel electrophoresis of RT-PCR amplified products (231bp CBSV coat protein gene fragment) using CBSV-specific primer pair CBSV 10F and CBSV 11R. RNA isolated from different parts of CBSD-affected plants cv. Albert: “L”, 1kb plus DNA marker; 1- non-necrotic root tissue from diseased plant, 2- peelings from tender stem with necrotic

lesion, 3 – young tender leaves, 4 – flowers, 5- fruits, 6- youngest symptomatic leaves, 7- mature leaves with clear symptoms, + - positive control, 8- seed from infected plant.

Virus was detected in almost all live tissues tested except in seeds, and peelings from the middle and mature sections of the stem. CBSV was not detected from necrotic tissues of the roots. Brighter bands were apparent in samples from non-necrotic root tissues, young tender leaves and the youngest symptomatic leaves.

Repeated assessment (in triplicate) of the proportionate distribution of extractable and detectable virus in plant tissue in three other cultivars, Cheupe, Kibaha and Nachinyaya gave results similar to that of Albert (Table 4.6). CBSV was detected in flowers, fruits, apical buds, young tender leaves, newly open leaves, youngest symptomatic leaves, tender-top green stem portions and non-necrotic storage root tissues. CBSV was also detected from non-necrotic storage root tissues obtained from cv. Nachinyaya which only exhibited foliage symptoms.

Table 4.6 CBSV detection in plant parts of CBSD-affected cassava plants

*Plant parts tested	*Tested cultivars and band strength			
	Albert	Cheupe	Kibaha	Nachinyaya
Flowers	+	+	+/-	+
Fruits (symptomless)	+	+	+	+
Seeds	-	-	-	-
Apical buds	++	++	++	++
Young tender leaves (symptomless)	++	++	++	++
Newly open leaves (symptomless)	++	++	++	+
Youngest symptomatic leaves	++	+	++	++
Mature leaves (clear symptoms)	+/-	+/-	+/-	+/-
Senescent leaves	-	-	-	+/-
Tender, top green part of the stem	+	+	+	+/-
Mid-stem peelings	-	-	-	-
Bottom woody peelings	-	-	-	-
Necrotic root tissue	-	-	-	-
Necrotic margin tissues	-	-	-	-
Clear white non-necrotic cortical tissue	++	++	++	++

*CBSV was detected from different plant parts collected from four CBSD-affected cultivars collected from field plants at SRI. Annotations to RT-PCR band strength are; -, negative (no amplification band), +, normal clear band, ++ very clear band, +/- band present but not clear.

Table 4. 7 Regeneration of cassava plants from CBSV-infected cuttings of cvs. Albert, Cheupe and Nachinyaya

			Mean severity score and % of symptomatic plants						RT-PCR
			2MAP		3MAP		4MAP		
Variety	Score at planting*	Test plants	Score	% symptomatic	Score	% symptomatic	Score	% symptomatic	CBSV-free (%)
Albert	2	15	2.0	67	3.6	67	3.7	67	4.4
	3	15	2.8	100	3.2	100	3.6	100	0
	4	15	3.6	100	3.8	100	4.2	100	0
AR 49/2	3	20	3.8	100	3.8	100	4.3	100	0
	4	25	4.0	100	4.2	100	4.8	100	0
Cheupe	2	15	2.9	93	3.1	100	3.4	100	0
	3	15	3.6	100	4.0	100	4.8	100	0
	4	15	3.6	100	4.0	100	4.8	100	0
Nachinyaya	2	15	2.4	75	2.6	92	2.9	100	0
	3	15	2.4	73	2.9	87	3.7	100	0
	4	15	3.0	87	3.3	100	3.3	100	0

All the cuttings tested in table 4.6 were obtained from CBSD-affected mother plants and CBSV confirmed by RT-PCR. Five cuttings were collected from each of the top green, middle part and bottom woody portion of the stem. Cuttings from each stem section were planted in separate pots. A total of 45 cuttings for each variety were established in pots in the screenhouse and the rate of symptom development monitored for four months.

* Indicates severity score of the mother plant from which the cuttings were derived. The disease-free percentage is calculated as the proportion of the total number of the plants assessed per cultivar.

4. 4. 9 Regeneration of healthy plants from CBSD-affected cuttings

With the exception of Albert, none of the potted cuttings taken from the four diseased cultivars' stems sprouted disease-free (Table 4.7). Foliage blotches and vein chlorosis were the major symptoms observed. Five of 15 (33 %) cuttings from cv. Albert (data not shown), sprouted symptomless despite being derived from CBSV-infected mother plants with severity score 2 and confirmed to contain CBSV by RT-PCR. The symptomless plants emerged irrespective of the stem portion from which the cuttings were obtained. Three symptomless plants were from the top-green portion and the other two were from the mature bottom portion of the stem. Triplicate tests by RT-PCR detected CBSV in two of the five (40 %) symptom-less plants.

4. 5 DISCUSSION

Three major types of foliage and root symptoms in CBSV-infected cassava were delineated which included chlorotic blotches, chlorotic spots and veinal chlorosis. The occurrence and development of foliage chlorotic blotches and veinal chlorosis were similar to Nichols' (1950a) and Storey's (1936) original descriptions. The only difference was the nature of symptom development in veinal chlorosis. Most often chlorosis started in tertiary veins but not the secondary veins and later expanded to cover the secondary and primary veins. Blotches were the most frequent symptom. Chlorotic spots were a newly observed CBSD-symptom which has not been described previously. CBSV was detected in leaf samples exhibiting all three major types of symptoms. The three types of foliage symptom were observed throughout each of the three surveyed Zones of Tanzania. Senescing CBSD-affected leaves always retained some green

areas while CBSD-free leaves appeared uniformly yellow. This observation conformed to previous reports (Hillocks and Thresh, 1998; Nichols, 1950a).

Other newly observed CBSD symptoms included: the occurrence of dark-brown necrotic tissues impregnated in the bases of leaf petioles, irregular senescence and poor leaf retention in some cultivars. The cause and nature of necrotic tissues in leaf petiole bases could be similar to root necrosis. Formation of tyloses was suspected to be the cause of the three symptoms observed. According to Agrios (2005), some plants when invaded by pathogens, secrete hormones that trigger the development of finger-like tissues termed tyloses in xylem to restrict further invasion of unaffected tissues. Consequently this causes blockages of water and nutrient movement resulting in wilting and death of plant parts deprived of nutrients and water.

The virus-induced changes in plant cell function could have caused the occurrence of the irregular senescence and poor leaf retention. Ruberson (1999) reported that soon after infection, some viruses disrupt the normal plant metabolism by taking over the plant cell nucleic acid replication, and redirecting it to functions that are detrimental. However, experimental evidence would be required to justify these assumptions.

The cortical root symptoms included the brown necrotic masses, necrotic specks and chalky necrosis. Appearances of the two types (brown necrotic masses and necrotic specks) were consistent with the observation of Jennings (1957), Nichols (1950a) and Storey (1936). Chalky necrosis has never previously been described. Interestingly, most plants that exhibited the chalky

type of root symptom rarely developed foliage symptoms. The chalky-white substances are suspected to be the polysaccharides often deposited in some of the plant tissues to prevent further spread of the virus (Ruberson, 1999; Matthews, 1991). Thus, chalky necrosis could suggest a form of plant reaction to CBSV-infection in resistant cultivars. However, detailed studies will be required to confirm this suspicion.

Expression of foliage symptoms of CBSD was found to be suggestive of CBSV infection but not conclusive of the presence of the virus. Some plants with apparent CBSD foliage symptoms (22 %) were diagnosed virus-free while 7 % of leaf samples collected from symptom-less plants tested positive for CBSV. Detection of CBSV from apparently symptom-less plants could be explained by abscission of the older CBSD-symptomatic leaves of the CBSD-affected plants often experienced during the dry seasons. Most of the symptomatic but CBSV-negative samples were recorded from the Lake zone. Failure to detect CBSV in apparently symptomatic plants indicates that symptoms observed were caused either by another pathogen or some other abiotic factors such as mineral deficiency. Deficiency of nutrients elements such as phosphorus, magnesium, potassium, iron and zinc have been reported to cause abnormal colouration, discolouration and/or death of leaf tissues (Matthews, 1991). Deficiencies of magnesium and iron have been reported to cause yellowing and necrosis in sugar beet similar to that of *Beet yellows virus* (BYV) (Uchida, 2000; Wallace, 1943). In potato, potassium and magnesium deficiencies cause marginal and interveinal necrosis similar to viral symptoms (Matthews, 2002).

Another possibility is that, the high environmental temperatures could have induced physiological stress causing chlorosis and a sort of vein clearing that were misidentified as

CBSD symptoms in some plants. This suspicion is based on the fact that the survey was conducted during the dry season from August to October when temperatures in Tanzania are relatively high. Most cuttings obtained from the CBSD-symptomatic but CBSV-free plants by RT-PCR, did not exhibit the disease symptoms upon regeneration in pots in the screenhouse. Similar observations on the effect of temperature stress were reported in *Nicotiana glutinosa* plants (John and Weintraub, 1966). When held at 37.8 ^{0}C for 4-8 days the new in *N. glutinosa* plant leaves displayed a pattern of mosaic, vein clearing, chlorosis and other abnormalities which resembles those caused by virus infections.

Non-uniform distribution of CBSV in sampled leaf tissue may also have caused non-detection of CBSV in symptomatic tissues. Atkins and Matthews (1970), reported on the variation in distribution of virus particles based on mosaic pattern in tobacco plants. In an experiment on the distribution of *Tobacco mosaic virus* (TMV) in a tobacco leaf, it was established that the dark green areas contain very little detectable virus particles compared with the yellow or yellow green areas. A similar phenomenon could occur with CBSD-affected plants in that leaf portions used for CBSV detection were more of the green portion compared to the yellow portion of mosaic.

Latent infection with CBSV may be a feature of tolerant cultivars as reported for other pathosystems (Matthews, 2002). Plants co-existence with infective pathogens was described as a mechanism of adaptation (Lerner, 1999). Despite such occurrences, the majority of CBSV-positive samples (67 %) were symptomatic. Some of the symptomless plants found to be CBSV-

infected could have expressed CBSD symptoms at an earlier stage of growth or might have done so later.

CBSV was detected from non-necrotic cortical root tissue, peelings from tender stem, young tender leaves, flowers, fruits, youngest symptomatic leaves and mature leaves with clear symptoms. This suggests that most portions of infected plants can be used to sample for CBSV. Based on band strengths, however, it was apparent that non-necrotic cortical root tissue, young tender leaves and youngest symptomatic leaves offers the best sample for the RNA isolation technique used. These observations are consistent with classical studies on virus distribution in plants (Samuel, 1934) in which upon inoculation, TMV was found to move from the point of inoculation to the root system first, successfully infecting the youngest leaves and thereafter, the older leaves. In addition, the presence of limited amounts of phenolic compounds in young tissue (Lerner, 1999) could also explain these observations. Phenolic compounds in plants usually increases with plant age and may sometimes interfere with nucleic acids extraction reagents (Barnett and Fulton, 1971). The detection of CBSV in root tissue provides a basis for separating root necrosis due to CBSD and that caused by invasive facultative soil-inhabiting fungi species reported by Hillocks (1997).

CBSV was not detected in seeds, mature stem peelings or necrotic root tissue. This again suggested an uneven distribution of virus in infected plants as recorded with other pathosystems (Matthews, 1991). The absence of CBSV in seeds obtained from diseased plants was consistent with findings of Maruthi *et al.*, (2005). The detection of CBSV in flowers and fruits of CBSV-infected plants but not in seeds suggests the possible existence of mechanisms that excludes the

virus from the embryo as reported by Carroll, (1972) from experience with other viruses. This provides an opportunity to obtain CBSV-free plants from seed. Failure to detect CBSV in peelings from infected stems could suggest that CBSV is more likely to be contained in xylem and phloem tissues compared to the epidermal tissues. The comprehensive study conducted to assess whether mature stem parts contain CBSV although the virus was not detected in peelings confirmed the presence of the CBSV in mature portions of cassava stems.

The inefficiency of the RNA extraction reagents used may account for the failure to detect the virus in stem peelings. According to Barnett and Fulton (1971), the method used to extract virus from host tissue may frequently leave a variable but quite large proportion of virus bound to cell debris or retained in vesicles or organelles. Some viruses, particularly the rod-shaped ones that occur as large fibrous inclusions in the cell, may remain aggregated during the initial stages of isolation (Matthews, 1991). Stem tissues of most plants are known to contain lignified compounds and cellulose (Lerner, 1999). Virus extraction from these lignified compounds may be difficult causing unavailability or limited amount of detectable viral RNA (Matthews, 2002). Based on these results, it was established that stem peelings do not provide good samples for CBSV detection.

In contrast, CBSV was neither detected from the necrotic tissue nor the necrotic margin of the root cortex. This could be explained by the obligate nature of viruses (Matthews, 1991). The necrotic portions of plant tissues are usually comprised of dead plant cells which may not support the survival of viruses (Agrios, 1997). This observation further

confirms the observations described in Chapter 3, that CBSV may not be transmitted through infected root debris because the virus does not survive in dead tissues.

The non-uniform distribution of CBSV in the mother plants from which the cuttings were obtained may account for the occurrence of a few CBSV-free plants. Some symptomless plants sprouted from cuttings obtained from the bottom and middle stem sections of CBSD-affected plants with mild foliage symptoms (score 2). Historically, the mother plants from which the cuttings were obtained were originally CBSV-free as developed through tissue culture and later became infected in the field. Due to the low CBSD score, the plants were suspected to have been infected recently with localised distribution of the virus. Maule, (1991) reported that the final distribution of virus (after successful infection) through tissue and organs may be very uneven and it may take a long time to become uniform. It is suspected that CBSV had not spread throughout the plant systems.

There could have been a tendency to recover from CBSV infection as described with *Cassava mosaic geminiviruses* (CMGs) (Fargette *et al.*, 1996). Observations in this experiment suggest that more work is required with more cassava cultivars, particularly the CBSV-resistant ones to affirm the suspected mechanism of field resistance to CBSV.

Although a few CBSV-free plants regenerated from CBSD-affected plants, it was generally shown that disease-free plants are not obtained from infected mother plants. The claims given by farmers that disease-free plants could be regenerated from infected mother

plants could have been based on poor diagnosis of CBSD symptoms. As shown previously, 22 % of sampled plants had foliage symptoms similar to those of CBSD but tested negative to the virus, the fact which triggered suspicion of the abiotic factors to have caused the symptoms.

The findings from this study suggest that CBSD symptoms are diverse and highlight the difficulty of using leaf symptoms alone to diagnose CBSV infection. Careful observations are required to correctly diagnose the disease. Symptom-based diagnosis of CBSD is less reliable than the molecular techniques. Hence, the need for robust and affordable diagnostic tests cannot be overemphasized. Molecular diagnostic techniques should always supplement the symptom-based CBSD diagnosis. The new types of CBSD foliage and root symptoms delineated may improve on the diagnostic efficiency. For the first time, this study has established the relationship between symptom expression and CBSV presence in CBSD-affected plants. It was further established that expression of CBSD foliage symptoms may not necessarily indicate the presence of the virus. Moreover, not all symptomless plants are free from CBSV. The detection of the virus in symptomless plants complicates its diagnosis. Consequently disease management techniques such as phytosanitation and quarantine have limited role to play in counteracting further spread of CBSD.

CHAPTER 5

FACTORS AFFECTING DISEASE INCIDENCE AND SEVERITY IN CASSAVA BROWN STREAK-AFFECTED CASSAVA PLANTS

Manuscript in preparation for submission to Journal of Plant Pathology

5.1 SUMMARY

Manifestation of CBSD infections in susceptible cassava cultivars is usually through symptoms. The disease symptoms are often expressed in leaves, stem and roots but little is known about the factors that affect symptom expression. In the current study, a series of experiments was conducted in the field, screenhouse and plant growth chamber to investigate factors affecting disease incidence and severity in CBSD-affected cassava plants. The inherent cultivar characteristics and plant growth stage had influence on the incidence and severity of CBSD in CBSV-infected plants. Foliage and stem CBSD incidences and severities were significantly ($P < 0.001$) related to the individual cultivars. The effects of plant age on CBSD incidences and severities were also significant ($P < 0.05$). Highest CBSD incidences and severities were recorded starting at nine months to twelve months after planting. Dual infections of CBSD and CMD were recorded in four of the six test cultivars. The numbers of *B. tabaci* was negatively correlated (*r*) with CBSD indices. For the first time, the study established the critical levels for cool temperatures (26 ^{0}C day and 18 ^{0}C night temperature) that are lethal to CBSD-affected plants and experimentally confirmed the long suspected influence of temperature and moisture stresses on CBSD. Exposure of CBSD-affected plants of cvs. Albert, Cheupe and Nachinyaya to these temperatures led to the death of more than 50 % of test plants within 30 days but none of disease-free (control) plants died. Moisture stress led to more than half of the CBSD-affected test plants dying within 90 days.

5. 2. INTRODUCTION

Cassava brown streak virus (*Ipomovirus; Potyviridae*) (Monger *et al*., 2001a) often infects susceptible cassava (*Manihot esculenta* Crantz) in some parts of eastern and southern Africa to cause cassava brown streak disease (CBSD). Following infection, the virus triggers development of symptoms, usually considered indicative of the disease in diagnosis. Storey (1936 & 1939) first reported the disease symptoms but more comprehensive descriptions were provided subsequently by Hillocks and Thresh, (1998) and Nichols, (1950a).

Variability in symptom expression among CBSD-affected cultivars when grown in different environments has been reported (Munthali, 1992). This posed a question as to what exactly determines the incidence and severity of CBSD. Several reports (Hillocks and Jennings, 2003; Hillocks *et al*., 1996; 2001; Jennings, 1957; Nichols, 1950a) suggested several factors contributing to the severity of CBSD in susceptible plants. These included: environmental conditions (altitude and temperature), crop growth stage relative to the time of infection and cultivar sensitivity. However the critical levels of temperature that affect severity of the disease have never been determined. There are no reports on the dynamics of the disease throughout the growth stages of diseased plants. The response of different cassava cultivars to CBSD across seasons is also poorly documented. In addition, Nichols, (1950b) suspected that drought aggravates the severity of the disease but the claim has not been substantiated.

The current study was conducted to determine the factors affecting incidence and severity of CBSD. The study specifically aimed at; (i) determining the susceptibility of selected cultivars, (ii) examining disease progress with respect to the age of the infected plants, (iii) determining the

effect of temperature on affected plants, and (iv) examining the effect of moisture stress on affected plants.

5. 3 MATERIALS AND METHODS

Various experiments were conducted in the field, screenhouse and plant growth chamber to determine the factors that influence symptom expression and severity in CBSD-affected plants. A field experiment was conducted to determine the response of six CBSD-affected cultivars to the disease and the influence of cultivar characteristics and plant growth stage on CBSD incidence and severity. Other experiments were conducted in a plant growth chamber to determine the effect of temperature on CBSD-affected plants. In the screen house, the effect of water stress on CBSD-affected plants was also established.

5. 3. 1 Field experiment

A field experiment was conducted in a randomised complete block design at SRI-Kibaha, Tanzania in two consecutive seasons, June 2006/July 2007 and July 2007/August 2008 to determine the susceptibility of selected cultivars to CBSD. Six cultivars with different levels of sensitivity to CBSD namely: Albert, AR49/2, Cheupe, Kibaha, Nachinyaya and Namikonga were planted in three replicates. Hard-wood stem cuttings (25-30 cm long) were obtained from CBSD-affected plants (symptom severity score 2-5) of each of six cultivars and planted in a 6 m x 6 m plot at 1 m x 1 m spacing per cultivar per replication. Each net plot comprised 36 plants in six rows, bordered on either side by six plants of a CBSD-tolerant cultivar, Namikonga. The overall

plot size was therefore 6 m x 8 m. The inter-plot space was 2 m and space between blocks (replications) was 3 m. A total of 108 plants were assessed per cultivar per season.

Monthly assessments were made of foliage and stem incidence and severity of CBSD in the net plots of each of the six cultivars. Disease assessments were based on observable CBSD symptoms on leaves and stems. A scale of 1 (no symptoms) through 5 (severe symptoms) for CBSD assessment (Hillocks and Thresh, 1998) was used with minor modification as in Chapter 2, Table 2.1. CBSD severity was calculated based only on plants with scores of 2-5. In plants that became dually infected, the disease severity of CMD was also assessed as per Hillocks and Thresh (1998). Numbers of adult *B. tabaci* were also recorded monthly from five fully open top leaves on each test plant. Experimental plants were allowed to grow for twelve months after which they were all uprooted and used to provide cuttings for a second season. CBSD incidences and severity with respect to individual cultivars and time (growth stage) were the basic parameters for the experiment.

Data were analysed in the GenStat 4.24DE statistical package (Lawes Agric. Trust, UK). Analysis of variance (ANOVA) was performed to determine responses of test cultivars to CBSD based on foliage and stem incidences and severities of the disease. The mean values of observations were separated using Duncan's multiple range tests.

5. 3. 2 Effect of temperature on CBSD-affected plants

Two-month-old potted plants established from CBSD-affected cuttings of cultivars Albert, Cheupe and Nachinyaya were exposed to two different temperature regimes in a plant

growth chamber (LEEC, LT601L/RH, UK). Before exposure, all the CBSD-symptomatic leaves were removed to retain only the symptomless ones. Relative humidity in the plant growth chamber was set at 70 %. The temperature regimes at which the plants were tested were: 26 ^{0}C day and 18 ^{0}C day night temperature (lower range), and 32 ^{0}C day and 26 ^{0}C night temperature (higher range). Ten CBSD-affected and one CBSV-free plant(s) for each cultivar were tested at each temperature range.

Observations on all plants were done at ten-day intervals for 50 days. Symptom expression and other morphological or physiological features that developed on diseased plants in contrast to the controls were recorded. During each assessment, the incidence and severity of the disease were estimated. Relative quantifications were made of the size of chlorotic veins, blotches and spots. On each leaf, the diameter or length of chlorosis for smallest and largest blotches, spots and veins were measured in millimetres using a ruler. The proportion of leaf surface area covered by chlorosis relative to the green area was estimated visually. The proportions of chlorotic tertiary, secondary and primary veins were also estimated. In addition, the number of newly-formed leaves, dead leaves and dead plants were also recorded. The observed data were summarised and presented as graphs and tables. The disease progress curves were generated and the relationship equations on the plant response for each of the cultivars to the two temperature regimes were established.

In addition to the experiments conducted in the plant growth chamber, the disease parameters collected in the field experiment (section 2.1 above) were analysed in relation to

weather data collected from a meteorological facility located nearly 100 metres from the field. Temperature data were used in correlation and regression analyses in GenStat 4.24DE.

5. 3. 3 The effect of moisture stress on CBSD-affected plants

A pot experiment was conducted in the screenhouse at MARI to determine the effect of moisture stress on CBSD-affected and CBSV-free plants. CBSV-free plants were obtained previously through meristem tissue culture, tested and confirmed to be CBSV-free and thereafter maintained in the screenhouse at SRI, Kibaha. Three cultivars: Albert, Cheupe and Nachinyaya were tested. Pots of one litre capacity were filled with sterile loam soil to about 80 % volume. Cuttings (25 cm long) collected from CBSD-affected plants in the field and CBSV-free plants in the screenhouse were established in pots and left to grow for 50 days. Twelve CBSD-affected plants and six CBSV-free plants were selected from each cultivar for the water stress experiment. The CBSD-affected plants were further divided in two groups each of six plants. The moisture stress treatments were as follows: (1) CBSD-affected and well irrigated (50 mls distilled water applied per pot per day), (2) CBSD-affected and water stressed (5 mls distilled water applied per pot per day) and, (3) CBSV-free plants and water stressed (5 mls distilled water applied per pot per day). Six plants were allocated to each treatment. The numbers of withering and dying leaves were recorded at 10-day intervals for 90 days. The experiment was repeated three times at different times in the year 2007. The recorded numbers of dead leaves were averaged to obtain mean counts. Regression analysis was used (GenStat 4.24DE) to assess the relationship among tested cultivars and their response to water stress.

The daily water requirements for the potted plants were pre-determined in a pilot study using varied amount of water application of 2, 4, 5, 7.5, 10, 15, 25, 40, 50, 75 and 100 mls per day. In this screenhouse experiment, three plants were supplied with each of the water amounts for 25 days. The lowest threshold was established to be 4 mls, while 50 mls was the optimum. In the 4 mls treatment, plants had reduced growth while in the 75 mls treatment the pots became water-logged.

5. 4 RESULTS

5. 4. 1 Seasonality of CBSD incidence and severity in field-grown experimental plants

Foliage and stem incidences of CBSD were relatively higher in 2006/2007 than in 2007/2008 (Table 5.1). Stem symptoms were less common than foliage symptoms. CBSD incidence and severity varied significantly ($P < 0.001$) among the test cultivars during the first and second planting seasons. The ANOVA for each disease test parameter is indicated (Appendix V, Table E4-E11). Apart from CBSD, the test cultivars were also affected by CMD. The symptoms did not seem to vary between the two seasons although they affected the apparency of CBSD leaf symptoms. The number of adult *B. tabaci* was greater in 2007/2008 season compared to 2006/2007 season.

Table 5.1 Mean field incidence and severity of CBSD, severity of CMD and *B. tabaci* recorded in six test cultivars for twelve months in 2006/7 and 2007/8.

CBSD test parameter	Mean ± Std Error	Mean square	F-value	P-value
2006/2007 experiment				
Foliage incidence (%)	55.5 ± 0.70	18227	84.1	<0.001
Foliage severity	3.3 ± 0.04	24	59.8	<0.001
Stem incidence (%)	14.8 ± 0.78	4976	22.8	<0.001
Stem severity	1.9 ± 0.04	17	27	<0.001
CMD severity	2.4 ± 0.07	40	226.2	<0.001
B. tabaci count	0.8 ± 0.09	11	7.8	<0.001
2007/2008 experiment				
Foliage incidence (%)	41.6 ± 0.64	16374	33.6	<0.001
Foliage severity	3.1 ± 0.02	39	74.8	<0.001
Stem incidence (%)	8.7 ± 0.96	1646	4.5	<0.001
Stem severity	1.6 ± 0.01	8	6.8	<0.001
CMD severity	2.4 ± 0.28	18	33	<0.001
B. tabaci count	4.0 ± 0.07	25	1.5	0.195

5. 4. 2 Effect of cultivar on foliage and stem incidence and severity of CBSD

The foliage and stem incidences and severities of CBSD in field-grown cassava were dependent on the respective cultivar. The ANOVA (Tables 5.2) suggested highly significant ($P < 0.001$) influences of each of individual test cultivars on the disease indices.

Table 5.2 ANOVA for the effect of test cultivars on CBSD, CMD and number of *B. tabaci* in 2006/2007 and 2007/2008.

Source of variation	DF	Mean square	F-value	P-value
CBSD foliage incidence (%)	5	17198	79.8	<0.001
CBSD foliage severity	5	34	90.4	<0.001
CBSD stem incidence (%)	5	2980	23	<0.001
CBSD stem severity	5	12	27.3	<0.001
CMD severity	5	18	33	<0.001
B. tabaci per plant (mean)	5	14	2.9	0.016

Duncan's mean separation tests also indicated significant variation in response to CBSD and CMD by the tested cultivars (Table 5.3). The cv. Namikonga was the least affected by CBSD while cv. AR49/2 remained the most affected throughout the two growing seasons. The cv. AR49/2 was least sensitive to CMD followed by cv. Albert but the other four cultivars were equally sensitive to the disease. Likewise, cv. AR 49/2 was the least preferred by the *B. tabaci* compared to the rest of test cultivars.

Table 5.3 Effect of test cultivars on the foliage and stem incidence and severity of CBSD, CMD severity and mean *B. tabaci* count per plant in 2006/2007 and 2007/2008.

	*CBSD F.inc. (%)	*CBSD F.sev.	*CBSD St.inc. (%)	*CBSD St.sev.	*CMD-sev.	**B. tabaci*
Albert	60.8 d	4.0 c	23.8 d	2.3 c	1.8 b	2.3 b
Namikonga	7.1 a	1.5 a	0.6 a	1.1 a	2.8 c	3.4 c
Kibaha	50.6 bc	3.4 b	16.0 b	2.2 bc	2.8 c	2.4 c
Nachinyaya	48.1 b	3.4 b	2.3 a	1.2 a	2.9 c	1.9 ab
Cheupe	56.4 cd	3.8 c	10.9 b	1.9 b	2.9 c	2.9 b
AR49/2	69.8 e	4.2 d	17.8 c	2.4 c	1.3 a	1.8 a

*Values followed by different letters in a column were significantly different as determined by using Duncan's test (P= 0.05). Description to abbreviations in a table: 'F.inc.' for foliage incidence, 'F.sev.' for foliage severity, 'St.inc.' for stem incidence and 'St.sev.' for stem severity score of CBSD. 'CMD' for cassava mosaic disease, '*B. tabaci*' for mean number of whitefly count per shoot.

5. 4. 3 Effect of time on foliage and stem incidence and severity of CBSD

The foliage and stem incidences and severities of CBSD on the affected plants were found to increase with time (Table 5.4). Despite the slight seasonal variations in CBSD severity, the general trend shows that the highest foliage and stem incidences and severity of the disease were recorded after ten months or subsequently. Variations in disease incidence and severity were observed at varying age of experimental plants. ANOVA (Table 5.5) suggested a highly significant influence of plant age on incidence and severity of CBSD.

Table 5.4 Effect of plant age on the foliage and stem incidence and severity of CBSD, CMD severity and mean *B. tabaci* count per plant in 2006/2007 and 2007/2008.

Plant age (months)	*CBSD F.inc. (%)	*CBSD F.sev.	*CBSD St.inc. (%)	*CBSD St.sev.	*CMD -sev.	**B. tabaci*
1	25.2 a	2.8 a	0.1 a	1.0 a	1.8 a	1.1 ab
2	45.5 bc	3.0 a	2.9 b	1.9 bcd	2.4 ab	1.2 ab
3	45.6 bc	3.2 ab	14.8 bcd	2.0 bcd	2.7 b	1.2 ab
4	41.3 b	3.2 ab	13.1 bcd	1.8 bcd	1.9 ab	1.3 ab
5	41.7 b	3.2 ab	12.8 bcd	1.8 bcd	2.5 ab	2.1 b
6	43.4 b	3.5 ab	13.2 bcd	1.8 bcd	2.3 ab	4.8 d
7	46.1 bc	3.3 ab	8.5 ab	1.7 b	2.3 ab	5.1 d
8	53.0 bcd	3.4 ab	8.9 abc	1.7 b	2.5 ab	3.2 c
9	54.3 bcd	3.5 b	9.1 abc	1.6 b	2.6 b	4.7 d
10	61.4 cd	3.9 b	19.1 d	2.4 d	2.5 ab	3.4 c
11	64.2 d	3.9 b	18.4 cd	2.1 bcd	2.5 ab	0.7a
12	63.9 d	3.9 b	21.6 d	2.3 cd	2.4 ab	0.6 a

*Values followed by different letters in a column were significantly different as determined by analysis of variance (ANOVA) using Duncan's test (P= 0.05). Description to abbreviations in a table: 'F.inc.' for foliage incidence, 'F.sev.' for foliage severity, 'St.inc.' for stem incidence and 'St.sev.' for stem severity score of CBSD. 'CMD' for cassava mosaic disease, '*B. tabaci*' for mean number of adult whitefly per shoot.

Table 5.5 ANOVA for the effect of time on CBSD, CMD and number of *B. tabaci* in 2006/2007 and 2007/2008.

Source of variation	DF	Mean square	F-value	P-value
CBSD foliage incidence (%)	11	2280	4.4	<0.001
CBSD foliage severity	11	3	2.3	0.011
CBSD stem incidence (%)	11	734	4.4	<0.001
CBSD stem severity	11	2	3.7	<0.001
CMD severity	11	53	21.2	<0.001
B. tabaci per plant (mean)	11	1	1.3	0.254

5. 4. 4 Linear correlations among CBSD, CMD and *B. tabaci*

The Pearson's linear correlation analysis suggested a significant positive relationship between foliage and stem incidences and severities of CBSD in all tested cultivars throughout the experiments (Table 5.6). Foliage incidence of CBSD had a strong correlation (r > 0.9) with foliage severity of the disease. Likewise, CBSD stem incidence and severity were positively correlated (r > 0.9). During field experimentation, some of CBSD-affected plants were concurrently affected by CMD. Unlike CBSD, the severity of CMD did not vary with seasons. The incidences and severity of CBSD and CMD were independent of each other. The correlation analysis could not signify the relationship between the two diseases. Insignificant negative correlations were observed between incidences and severity of CBSD and that of CMD severity and the number of *B. tabaci* per plant.

Table 5.6 Linear correlation analysis among incidences and severities of CBSD, CMD severity and mean number of *B. tabaci*

	F. inc. (%)	F. sev.	St. inc. (%)	St. sev.	*B. tabaci*	CMD
F. incidence (%)	**1.00**					
F. severity	0.91	**1.00**				
St. incidence (%)	0.61	0.61	**1.00**			
St. severity	0.60	0.57	0.90	**1.00**		
B. tabaci	-0.12	-0.11	-0.15	-0.17	**1.00**	
CMD severity	-0.28	-0.29	-0.29	-0.26	0.20	**1.00**

Description to abbreviations in a table: 'F.inc.' for foliage incidence, 'F.sev.' for foliage severity, 'St.inc.' for stem incidence and 'St.sev.' for stem severity score of CBSD. 'CMD' for cassava mosaic disease, *'B. tabaci'* for mean number of whitefly count per plant.

5. 4. 5 Effect of temperature on CBSD-affected plant

Varying responses were recorded in CBSD-affected plants of cvs. Albert, Cheupe and Nachinyaya when exposed to the two temperature regimes (Tables 5.7a and 5.7b). Disease symptoms were more severe at low temperatures (26 ^{0}C day and 18 ^{0}C night) than at high temperatures (32 ^{0}C day and 26 ^{0}C night). The control plants (CBSV-free) exposed together with the test plants at the two temperature regimes did not exhibit any of CBSD symptoms and none died, although their growth was retarded at the lower temperature. It was further noticed that initially symptomless CBSV-infected plants cv. X (based on previous detection in RT-PCR) developed foliage vein chlorosis typical of CBSD, 40 days after exposure to the low temperature.

Despite the varied response to temperature among the three cultivars, the general trend suggested that exposure to low temperature increased the severity of the disease. There were big increase in the number and size of blotches and chlorotic veins in the low temperature treatment over the 50-day period of exposure. The chlorosis in tertiary veins quickly expanded to cover the secondary and primary veins. Reduced development of new leaves and fast death of already developed leaves which culminated in death of the whole plant were also noted (Plate 5A).

The comparative responses of the three varieties tested revealed that cv. Albert was the most sensitive to CBSV at the lower temperatures compared to cvs. Cheupe and Nachinyaya (Figure 5.1). Deaths of all test plants cv. Albert were recorded within 50 days compared to the 80 % deaths in cv. Cheupe and 40 % in cv. Nachinyaya. CBSD incidence data suggest that cvs. Cheupe and Nachinyaya had gradual responses to the temperature. Unlike cv. Albert which almost attained 100 % CBSD foliage incidence in 20 days after exposure (DAE) to low temperature, 40 DAE were required for the two other cultivars to exceed 90 %. At higher temperature range, cv. Nachinyaya had the lowest foliage incidence compared to the other cultivars (Table 5.7b). In addition the numbers of newly-formed leaves were greatly reduced in cv. Albert compared to the cvs. Cheupe and Nachinyaya.

Table 5.7a Response of cvs. Albert, Cheupe and Nachinyaya at 26 ^{0}C day and 18 ^{0}C night temperature for 50 days in a plant growth chamber.

Test parameter		Response of test cultivars in 50 days at low temperature																				
		cv. Albert					cv. Cheupe						cv. Nachinyaya						cv. X (latency)			
Days after exposure	***C**	**10**	**20**	**30**	**40**	**50**	***C**	**10**	**20**	**30**	**40**	**50**	***C**	**10**	**20**	**30**	**40**	**50**	**20**	**30**	**40**	5
Incidence (%)	0	60.7	94.0	100	100	100	0	70.7	76.3	83.0	100	100	0	48.0	61.7	81.8	97.1	98.6	0	0	37.5	64.
Observed symptoms																						
*Blotches/spot (%)	0	32.7	37.3	37.6	27.3	**x**	0	23.6	31.2	26.6	12.3	19.5	0	8.4	9.7	7.9	6.6	8.4	0	0	0	0
*Blotches/spot diameter(mm)	0	7.4	14.0	20.0	27.0	**x**	0	6.6	12.5	20.8	25.4	27.6	0	2.3	4.1	8.2	13.8	20.7	0	0	0	0
*Chlorotic veins (%)	0	8.5	14.9	7.6	10.3	**x**	0	13.8	9.11	12.4	39	65.8	0	19.6	17.6	22.5	24.4	26.6	0	0	6.8	9.7
Nature of veinal symptoms																						
*Tertiary veins (%)	0	100	100	100	100	**x**	0	100	100	96.2	100	100	0	100	100	100	95.5	100	0	0	4.8	11.
*Secondary veins (%)	0	67.0	85.7	67.0	100	**x**	0	50.0	51.9	92.3	92.3	100	0	59.1	57.1	87.8	93.2	94.4	0	0	0.3	5.2
*Primary veins (%)	0	0	0	33.3	33.3	**x**	0	0	7.4	15.4	69.2	80.0	0	0	21.4	36.6	50	61.1	0	0	0	0.4
Mean severity score	1	2.8	4.0	4.7	4.8	5	1.0	2.7	3.5	3.9	4.7	5.0	1	2.6	2.5	2.9	3.5	4.0	0	0	2.0	2.2
Newly formed leaves	0	0	0.2	0	0.1	0	0	0	0.4	0.1	0	0	0	0	0.4	0.1	0.2	0.1	0.1	0.2	0.2	0
Leaf death (%)	0	0	38.0	84.2	95.0	100	0	0	0	15.3	75.0	90.0	0	0	0	2	21	42	0	0	0	0
Plant death (%)	0	0	30.0	70.0	90.0	100	0	0	0	0	50	80	0	0	0	0	0	40	0	0	0	0

*** C**: CBSV-free plants (negative control), *sub-unit of the main parameter (mean) assessed, **x** complete death of leaf and/or the experimental plant. Reduced plants growth was the only effect of temperature observed on CBSV-free plants of all three cultivars used as control. The latently CBSV-infected plants exhibited foliage symptoms 40 days after exposur to low temperature.

Table 5.7b. Response of cvs. Albert, Cheupe and Nachinyaya at 32 ^{0}C day and 26 ^{0}C night temperature for 50 days in a plant growth chamber.

Test parameter	Response of test cultivars in 50 days														
	Albert					Cheupe					Nachinyaya				
Days after exposure	**10**	**20**	**30**	**40**	**50**	**10**	**20**	**30**	**40**	**50**	**10**	**20**	**30**	**40**	**50**
Incidence (%)	68.5	64.2	70.5	68.9	75.5	65.1	65.0	65.1	68.4	74.3	61.0	52.5	48.7	50.7	60.8
Observed symptoms															
*Blotches/spot (%)	14.3	18.6	25.8	30.0	33.8	12.8	16.0	21.8	27.9	32.5	1.0	2.1	2.5	3.4	3.8
*Blotches/spot diameter(mm)	2.9	3.9	4.9	6.1	7.4	2.2	2.8	3.3	4.2	5.2	1.3	2.1	2.6	3.3	3.5
*Chlorotic veins (%)	0.7	1.3	2.9	3.8	4.8	7.5	2.4	4.7	6.8	8.6	9.6	13.7	19.3	25.3	28.1
Nature of veinal symptoms															
*Tertiary veins (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	94.9	95.7
*Secondary veins (%)	40	80	100	100	100	41.2	61.1	100	95.2	95.5	55.0	60.0	80.6	82.1	84.8
*Primary veins (%)	0	0	33.3	83.3	85.7	0	0	30.0	52.4	81.8	0	16.0	22.6	46.2	45.6
Mean severity score	2.6	2.6	2.9	3.0	3.2	2.5	2.6	2.7	3	3.2	2.5	2.5	2.7	3.2	3.2
Newly formed leaves	0.1	0.8	0.4	0.8	0.5	0.3	1.0	1.1	0.7	0.8	0.4	1.1	1.0	0.8	0.8
Leaf death (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plant death (%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* sub-unit of the main parameter assessed. None of CBSV-free plants developed symptoms

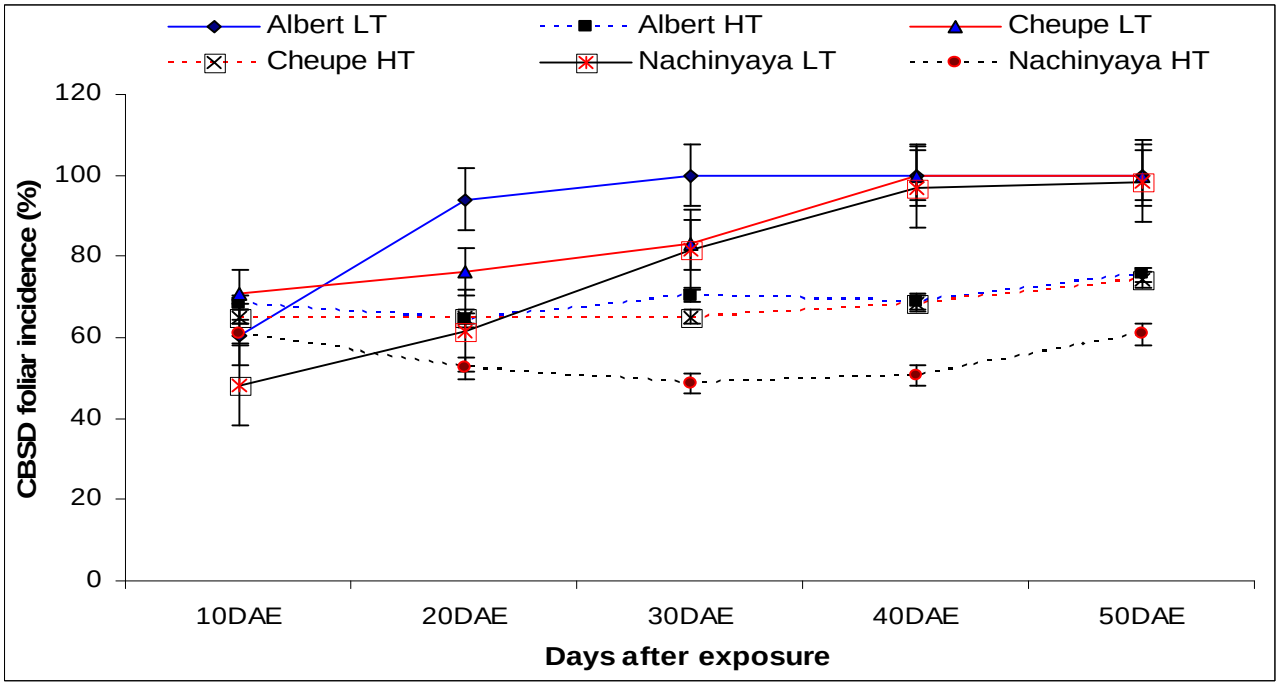


Figure 5.1. Comparative effect of temperature on foliage incidence of CBSD in three selected cultivars in a plant growth chamber: Day and night temperatures at 26 ^{0}C and 18 ^{0}C (low temperature-LT) presented as smooth lines and 32 ^{0}C and 26 ^{0}C (high temperature-HT) presented as dotted lines. Relative humidity was constantly set at 70 %.

5. 4. 6 Disease trend under controlled temperature

Increase in CBSD symptoms severity over time for the cvs. Albert, Cheupe and Nachinyaya under controlled temperature condition in the plant growth chamber are as presented in Figures 5.2, 5.3 and 5.4. All three cultivars exhibited severe foliage symptoms at low temperature range (26 ^{0}C day and 18 ^{0}C night) compared to the higher temperature range (32 ^{0}C day and 26 ^{0}C night). The Disease Severity increased with time at a decreasing rate at high temperature range. The regression analysis ($R^2 > 0.6$) in all test cultivars suggested a significant correlation between temperature regimes and disease severity.

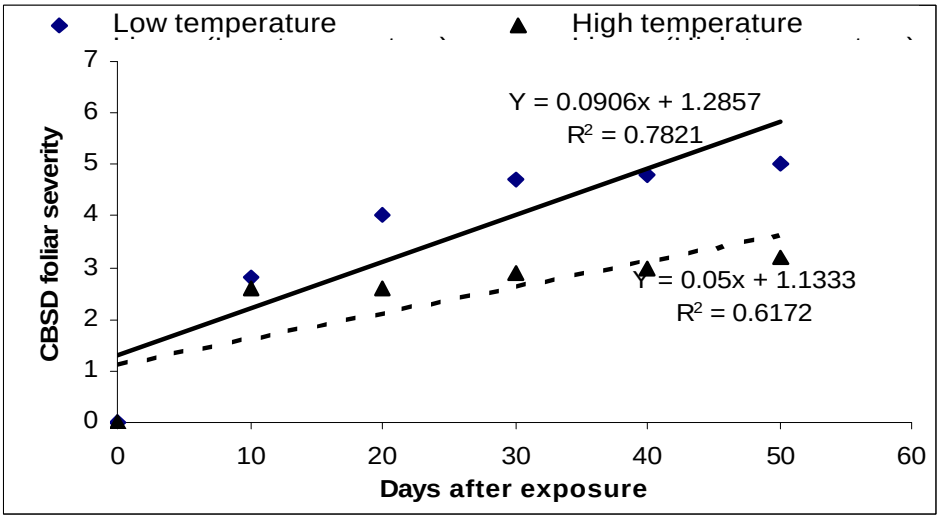


Figure 5.2 The disease severity trend for cv. Albert at 26 ^{0}C day and 18 ^{0}C night temperature and at 32 ^{0}C day and 26 ^{0}C night temperature. Note that a 1 to 5 severity scale was used as described previously. Score 1 is the lowest score and represents symptomless plants.

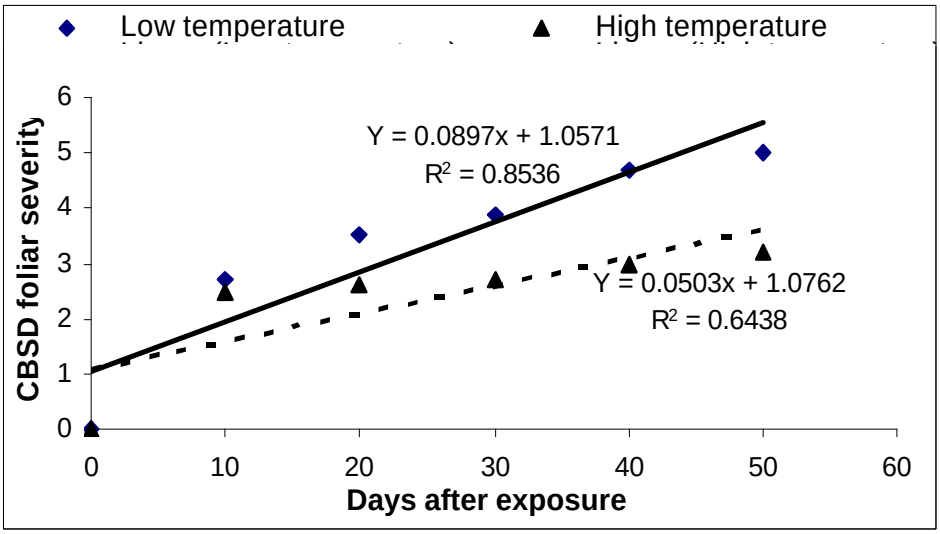


Figure 5.3 The disease severity trend for cv. Cheupe at 26 ^{0}C day and 18 ^{0}C night temperature and at 32 ^{0}C day and 26 ^{0}C night temperature. Note that a 1 to 5 severity scale was used as described previously. Score 1 is the lowest score and represents symptomless plants.

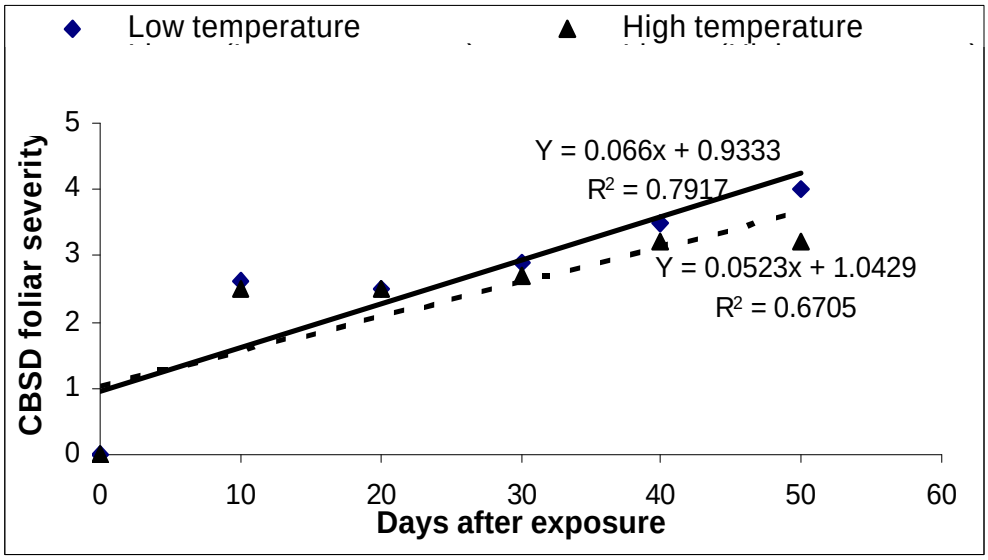


Figure 5.4 The disease severity trend for cv. Nachinyaya at 26 ^{0}C day and 18 ^{0}C night temperature and at 32 ^{0}C day and 26 ^{0}C night temperature. Note that a 1 to 5 severity scale was used as described previously. Score 1 is the lowest score and represents symptomless plants.

5.4.7 The effect of temperature on field-grown experimental plants

The foliage incidences of CBSD in the field-grown experimental cultivars were compared with the minimum and maximum temperature recorded from the nearby meteorological station by the Tanzanian Meteorological Agency. Unlike the observed effect of temperature under the controlled environment, limited influence of temperature on CBSD severity was observed in the experimental plants in the field (Figures 5.5a and 5.5b).

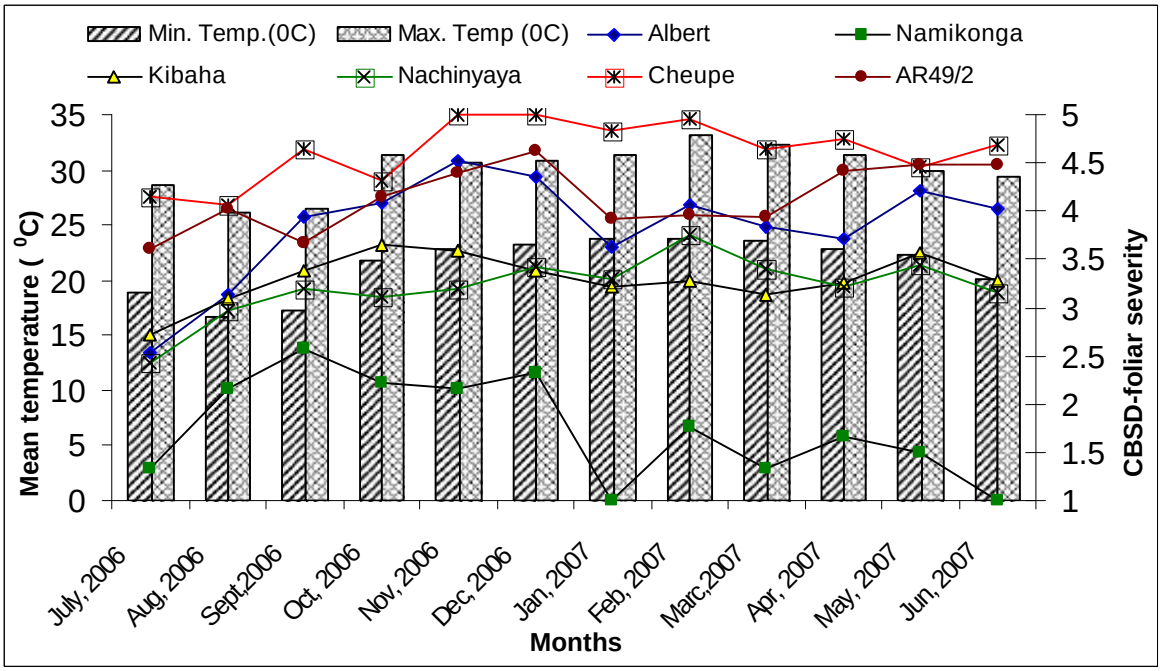


Figure 5.5a The relationship between field temperature and CBSD foliage severity in cvs. Albert, AR49/2, Cheupe, Kibaha, Nachinyaya and Namikonga for the season 2006/2007.

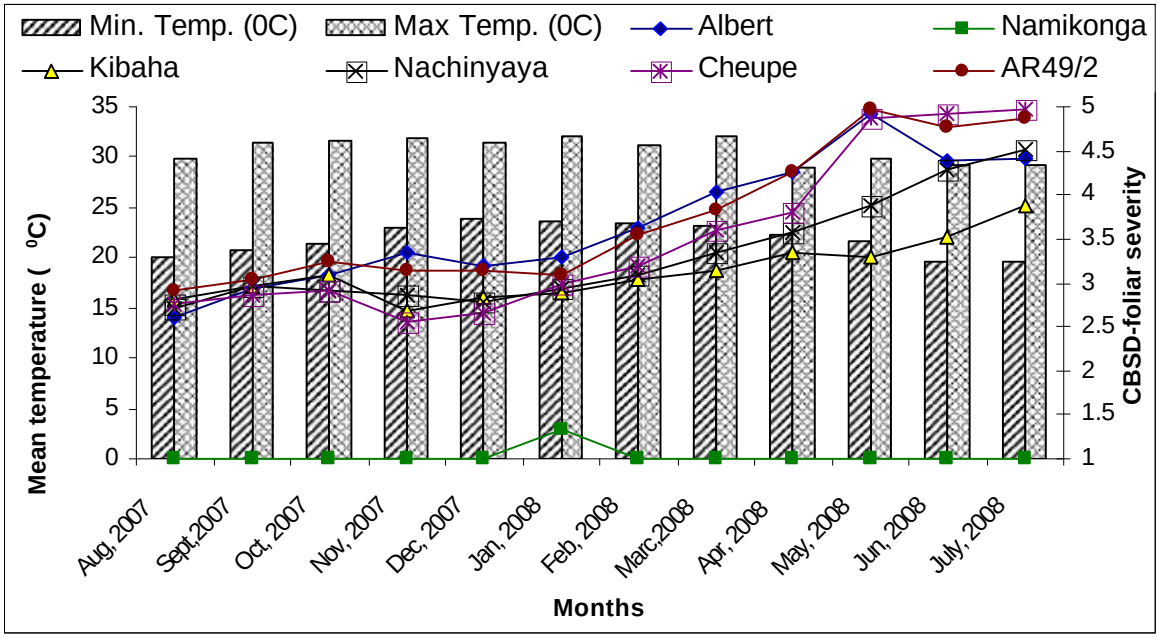


Figure 5.5b The relationship between field temperature and CBSD foliage severity in cvs. Albert, AR49/2, Cheupe, Kibaha, Nachinyaya and Namikonga for the season 2007/2008.

CBSD foliage severity varied in different months among test cultivars in the 2006/2007 and 2007/2008 experiments depending on the temperature changes. Consistently high severities were recorded in May and June 2007 when the minimum temperature was low at about 20^0C; and in May, June and July in 2007/2008 season, the months in which the lowest values of minimum temperature were recorded. Throughout the two growing season the mean monthly minimum and maximum temperature remained high at range of 19.6-24 ^{0}C and 25-35 ^{0}C, respectively.

5. 4. 8 Responses of CBSD-affected and CBSV-free plants to moisture stress

The most notable effect of moisture stress on CBSD-affected plants was wilting and death of leaves (Plate 5B). In all cultivars greatest leaf wilt was recorded in CBSD-affected moisture-stressed plants compared to the CBSD-free moisture-stressed plants (Table 5.8). The mean leaf deaths were 67 %, 54 % and 47 % for Albert, Cheupe and Nachinyaya, respectively.

Leaf deaths due to moisture stress in CBSD-affected plants started within 20 days of exposure and the number of dying leaves increased with time (Figure 5.5). At 90 days after exposure, almost half of the test plants had lost all their leaves and the stems had started to wither. Withering and death of leaves and stems were recorded in all six cultivars. A limited number of leaves (average of 2) died in cultivars Albert and Cheupe in the diseased well irrigated treatment. Neither leaves nor plants died in the CBSD-free, water-stressed treatment.

Table 5.8 Effect of moisture stress on leaf wilt (mortality) in CBSD-affected (D) and CBSD-free (H) plants for the cultivars: Albert, Cheupe and Nachinyaya.

	Albert			Cheupe			Nachinaya		
	D&S	D&IR	H&S	D&S	D&IR	H&S	D&S	D&IR	H&S
12DAE	0	0	0	0	0	0	0	0	0
20DAE	5	0.8	0	3.6	0.2	0	5.2	0	0
30DAE	20	0.5	0	17.6	0.5	0	12.3	0	0
40DAE	28.2	1.2	0	24.4	0.8	0	15.8	0.3	0
50DAE	27.2	0.9	0	31.1	1.0	0.1	26.0	0.6	0
60DAE	32.6	0.8	0	34.2	1.3	0.1	29.6	0.6	0
70DAE	39.8	1.1	0	35.9	1.3	0.2	34.0	0.6	0
80DAE	52.8	1.4	0	46.2	1.3	0.3	40.2	0.5	0
90DAE	67.0	3.1	0.5	54.0	2.4	0.4	47.0	0.7	0.1

Note: The tabular data presents average number of wilted (dead) leaves per six test plants; The abbreviation 'D' is for the CBSD-affected plants, 'H' for CBSD-free plants, 'S' exposed to moisture stress (5 mls per pot per day) and 'IR' normally irrigated (50 mls per pot per day). The plants were exposed to the treatment for 90 days.

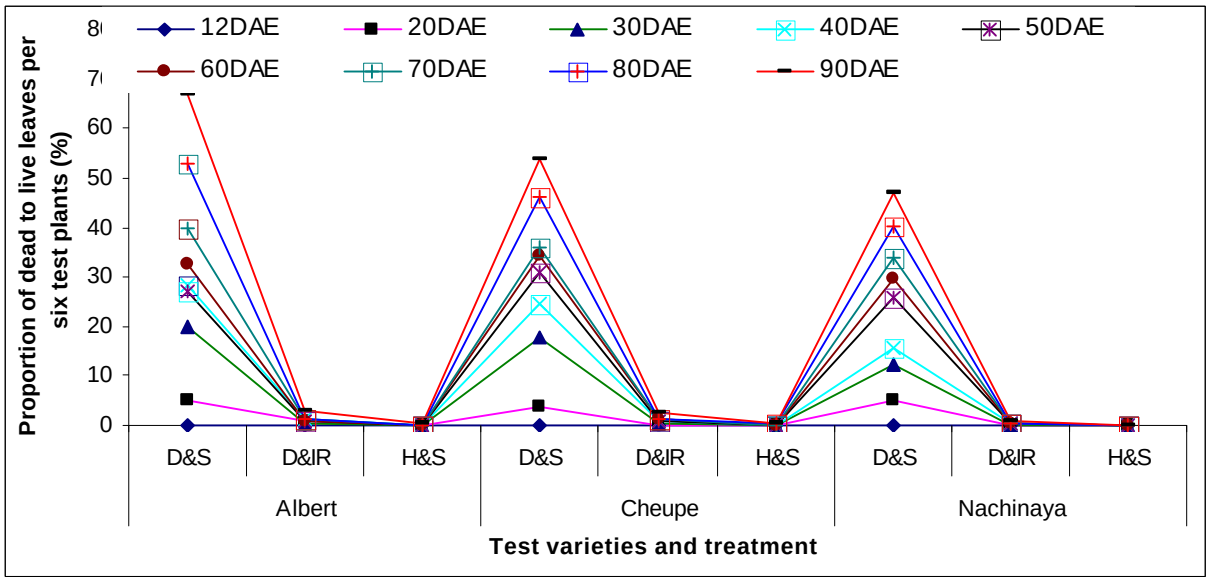


Figure 5.6 Effect of moisture stress on leaf death in CBSD-affected and CBSD-free plants for the three tested cultivars; Albert, Cheupe and Nachinyaya. The abbreviation 'D' is for the CBSD-affected plants, 'H' for CBSD-free plants, 'S' exposed to moisture stress (5 mls per pot

per day) and 'IR' normally irrigated (50 mls per pot per day). The plants were exposed to the treatment for 90 days.



Plate A Plate B

Plate 5A& B, Response of cassava plants cv. Albert to temperature and moisture stress.

Plate A; Dead cassava plant cv. Albert, 30 days after exposure to low temperature of 26^0C and 18^0C day and night temperature, respectively. **Plate B;** Comparison of plants of the same cv. Albert under different regimes of water application after 50 days of exposure. The abbreviation 'D' is for the CBSD-affected plants, 'H' for CBSD-free plants, 'S' exposed to moisture stress and 'IR' normally irrigated.

5. 4. 9 Regression analyses on the effect of moisture stress

The effect of moisture stress on the cvs. Albert, Cheupe and Nachinyaya was statistically significant ($R > 0.9$) in the regression analysis (Figures 5.7, 5.8 and 5.9). All the three test cultivars were affected similarly by the moisture stress.

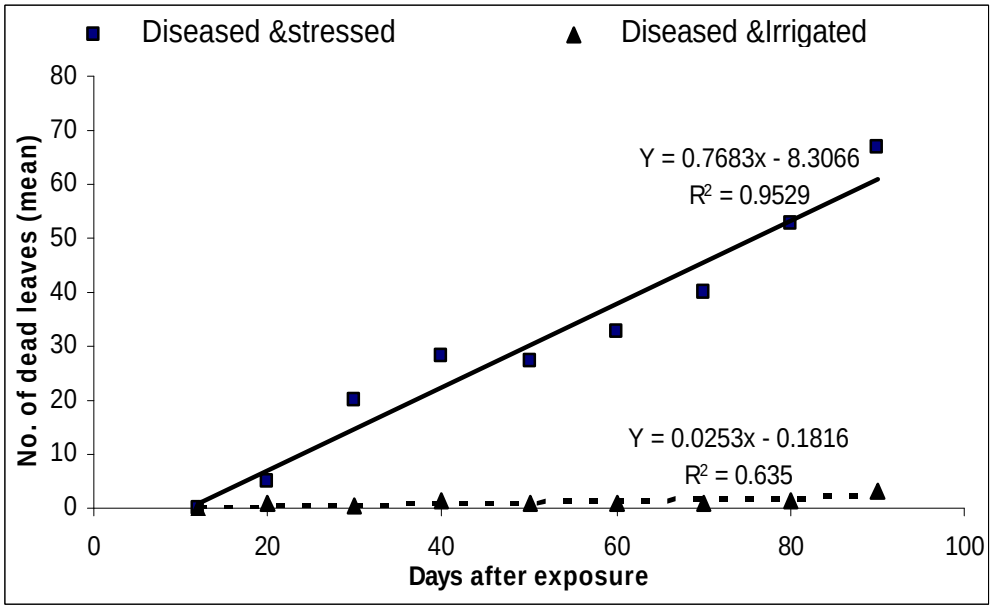


Figure 5.7 Relationship between leaf death and moisture stress in 90 days of exposure of the cv. Albert.

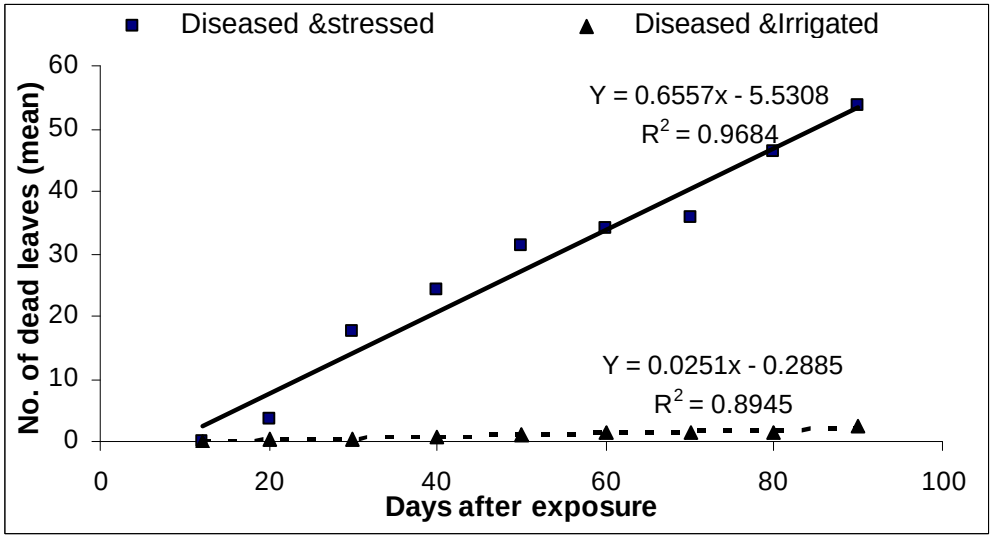


Figure 5.8 Relationship between leaf death and moisture stress in 90 days of exposure of the cv. Cheupe.

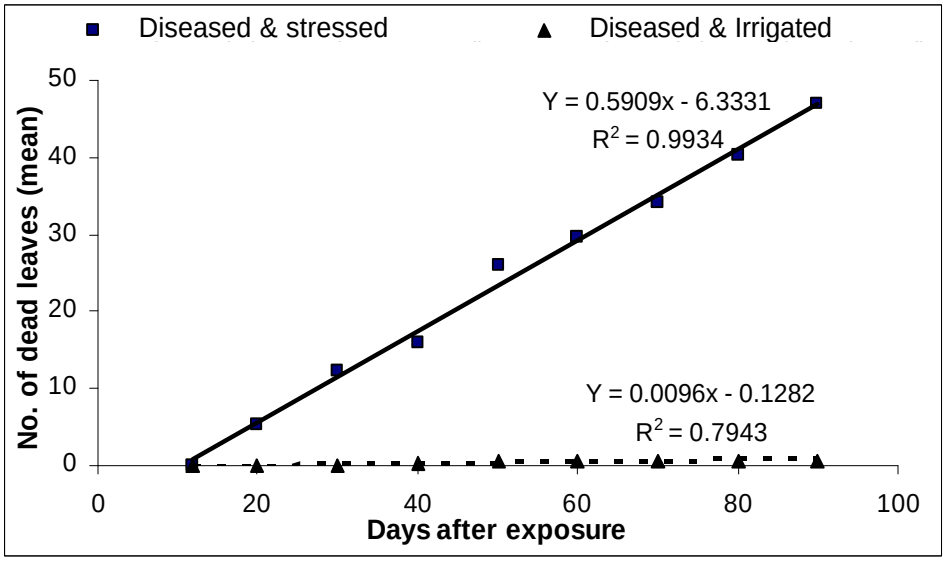


Figure 5.9 Relationship between leaf death and moisture stress in 90 days of exposure of the cv. Nachinyaya.

5. 5 DISCUSSION

Results of the field experiments presented here showed that CBSD symptoms were clearly expressed by leaves compared to stems and there were seasonal variation in stem symptoms. Similar observations have been reported previously (Hillocks, 1997; Hillocks and Jennings, 2003; Nichols 1950a). The observation confirmed that although CBSD was named after the stem symptoms, it is the foliage symptoms that are more likely to be seen and used for diagnosis.

Varied responses to CBSD were recorded among the tested cultivars in the field experiment. The incidence and severity of the disease was cultivar-specific. Throughout the experiments, cv. Namikonga exhibited the lowest incidence and severity of disease while cv. AR

49/2 was the most affected. Although all the experimental plants were obtained from CBSD-affected mother plants and grown in the same environment, severity was influenced by the inherent characteristics of individual cultivars. This observation is consistent with the Hillocks *et al.,* (2001) and Jennings, (1957) reports that different cultivars respond differently to CBSD.

The effect of time or plant age was apparent in all the cultivars tested. The foliage and stem incidences and severity of CBSD increased as plants grew. Despite some seasonal variations, the peak disease incidences and severities were attained at 10 months or later in all cultivars. The observed trend was somewhat related to the Hillocks *et al*., (1999) report that the levels of disease damages depends on the time for which plants are infected.

The effect of temperature on CBSD-affected plants was recorded when the day and night temperatures were set at 26 ^{0}C and 18 ^{0}C, respectively, in a plant growth chamber. Severe levels of CBSD and leaf or plant death were attained quickly at lower temperature compared to higher temperature. These seemed to be critical temperatures which were lethal to the diseased plants. The effect of such low temperature (< 19 ^{0}C) on CBSD incidence and severity was similarly reflected in field experimental plants particularly in 2007/2008. The temperature above these limits had insignificant effects on CBSD-affected plants. The effect of these critical levels of temperature clearly justified Nichols' (1950a) suspicion that the rare occurrences of CBSD-affected plants at higher altitudes were caused by lower temperature which adversely affected their survival. Jennings (1960) also reported that most CBSD-affected plants were killed during the cold season when extremely low temperatures were experienced. The findings from these experiments suggest that, locations characterised by cool temperatures in which the

environmental temperature consecutively falls below 18 ^{0}C for more than one month, presents ideal condition for massive testing for CBSV-infection particularly in breeding programmes for CBSD resistance.

CBSD-affected plants were more affected by moisture stress than CBSV-free plants. Leaf and plant death due to water stress were observed on CBSD-affected plants in all three cultivars tested. Clear contrast was seen between the diseased water-stressed and diseased well-irrigated plants. The response to moisture stress by withering and death of leaves and or the whole plant suggests that CBSD-affected plants are less protected from evapo-transpiration. CBSV-infection could have weakened the plants immunity, reducing their ability to withstand excessive evapo-transpiration and hence susceptibility to moisture stress. in contrast, blockage of water-conducting vessels could have increased the susceptibility of CBSD-affected plants to water stress. Lerner, (1999) indicated that plant exposure to water stress may trigger accumulation of polysaccharide materials blocking the xylem and disrupting water transport. In CBSD-affected plants, the response to water stress could have been fast and probably exacerbated by the formation of tyloses in the xylem to counteract CBSV spread. Such a scenario was reported with sugarbeet yellows disease in temperate Europe (Jaggard *et al.*, 1998), where plants infected with virus and subjected to drought stress suffered double the yield losses compared to the unstressed controls. The finding from this study has emphasized the significance of CBSD in exacerbating susceptibility to water stress of the affected plants. This confirms Nichols' (1950b) observation that severe incidences of CBSD were recorded in seasons during which drought stress prevailed.

The field occurrences of CBSD and CMD were independent of each other. Infection with either disease seemed not to affect the incidence and severity of the other. The major problem

noted however, was the difficulty in diagnosing CBSD symptoms in plants affected by both diseases. Similar observations were reported previously (Nichols, 1950a; Hillocks and Thresh, 1998; Hillocks and Jennings, 2003). The nature of interaction between CBSD and CMD could be more apparent at molecular level than the observable incidences and severity of the diseases on the affected plants. A detailed study may be required in future to elucidate the nature of interaction between the two diseases.

The findings from these studies have shown that foliage CBSD symptoms are more apparent compared to stem symptoms. The stem symptoms are very seasonal and cultivar-specific. Different varieties respond differently to CBSV-infection with varying incidences and severity of the disease. CBSD incidence and severity vary with plants age. Some cultivars are co-infected by CBSV and CMVs when grown in the field. CMD severity and numbers of *B. tabaci* may have limited influence on CBSD incidences and severities. Low temperature (26 ^{0}C day and 18 ^{0}C night temperatures) are critically lethal to the survivorship of CBSD-affected plants. Infection with CBSV increases the susceptibility of the CBSD-affected plants to moisture stress. In conclusion, the inherent genetically controlled response of different cultivars to CBSD, plant growth stage, environmental temperature and moisture are among the important factors that determine the disease manifestation in CBSD-affected plants.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

In this latest epidemiological research, the essential components of CBSD were studied and important biological facts about CBSD were established. The knowledge generated is considered fundamental in understanding CBSD and the nature of interaction between CBSV, susceptible cultivars of cultivated cassava and the environment in which plants survives. The information generated will guide the decisions in designing the effective management strategies for CBSD.

During the surveys, CBSD and its causal agent, CBSV were found in all cassava growing areas in Tanzania from 5 masl to 1900 masl. This is in contrast to the previous reports (Nichols, 1950a; Legg and Raya, 1998), that altitude had limited influence on the occurrence and distribution of the disease. The current widespread distribution of CBSD suggests that the disease will cause a national disaster and has serious implications for cassava production. Since cassava is regarded a food security crop in Tanzania, most households in cassava growing areas, particularly in Mara Region in the Lake Zone are destined for famine.

In addition, the demonstration of diverse CBSV isolates in Tanzania poses new challenges in understanding the nature of the disease. The diversity of CBSV isolates could be one of the causes for the variation in symptom expression, which was observed in diseased

plants. Possible synergies between CBSV isolates and the effect of cassava mosaic begomoviruses also need to be considered. Symptoms variation was previously attributed to the inherent genetotype of the respective cultivar (Jennings, 1960). However, based on current observations, the effect of CBSV strains on the way cassava cultivars respond to infections must not be overlooked.

Transmission and spread of CBSV was for a long time perceived to be solely through vectors and infected cuttings. Transmission of the virus by *B. tabaci* was demonstrated recently (Maruthi *et al.*, 2005). Sap and graft transmission of CBSV (Lister, 1959; Storey, 1936) were also confirmed. Agronomical practices are known to aid the transmission of other potyviruses that are easily transmitted mechanically. However, this has never been explored for CBSV and has been suspected to contribute to virus spread. In addition, the infectivity and efficiency of the reported non-vector methods of CBSV transmission have never been determined. This study established for the first time that CBSV may be transmitted through contaminated cutting tools and possibly the harvesting of cassava leaves for vegetable consumption. This suggested the possibility that cassava growers have unknowingly contributed to the new incidences and spread of CBSD through various agronomic practices. The appropriate formulations of CBSV inoculation buffers for mechanical transmission to cassava and conditions for sap inoculations were established whereby buffer B proved to be more effective than buffer A. CBSV transmission by grafting in which virus-free scion is grafted on infected root stock, was the most efficient mechanism. Up to 100 % of the recipient plants were infected within four weeks. The observation suggests that this grafting technique is the most effective one and may be used in rapid CBSV tests for screening purposes.

Early workers on CBSD described two major types of foliage and root symptoms (Nichols, 1950a; Storey, 1939). The current study identified three major types of foliage and root symptoms caused by CBSV. Spotty chlorosis is a new type of foliage CBSD symptom identified. About 31 % of all assessed samples had this type of symptom which accounted for 24 % of the symptomatic and CBSV-positive samples. Spotty chlorosis was a third type of CBSD foliage symptoms after the previously reported chlorotic blotches and veinal chlorosis. This could be the most overlooked symptom in the previous CBSD diagnostic surveys that were symptom-based.

Another symptom identified was the 'chalky white necrosis' (chalky necrosis) in the roots of CBSD-affected plants. This type of symptom was recorded in more than 19 % of the survey samples and accounted for 34 % of CBSV-positive symptomatic samples. Like the leaf symptoms, this was a third type of CBSD internal root symptoms described for the first time. Plants with chalky necrotic areas had mild or none of the foliage symptoms of CBSD. The identification and description of these new types of symptom may contribute to an improved diagnosis of the disease which is often symptom-based, and also serve as a basis for further investigation as to the cause of this unusual symptom phenotype. The sepia or brown necrotic tissue impregnated in the leaf petiole base, which expands to interfere with the abscission layer at senescence was another new symptom described for CBSD. Retention of the senesced leaves has been reported (Hillocks and Thresh, 1998; Hillocks and Jennings, 2003). However, the actual cause of failure leaf shed had never been identified. Although there might be other factors involved in leaf shedding, beyond the scope of this study, necrotic impregnation in the petiole base could be among them. This was a common symptom observed in leaves which failed to

shed after senescence. Poor leaf retention and irregular senescence of leaves were also the newly established symptoms of CBSD. One other possible cause could be the detrimental affects that CBSV has on the vascular system, as manifested by root symptoms. This would surely lead to irregular senescence and leaf shedding. The knowledge on all these field symptoms which are characteristic of CBSD, contributes to an understanding of CBSD etiology which is influenced by several factors..

The current study adopted a classical approach to study the relationship between CBSV infection and symptoms expression. It was further established and confirmed from this study that not all symptoms (particularly foliage ones that are apparently similar to Nichols (1950a) descriptions of CBSD, are caused by CBSV infection. Moreover, not all symptomless cassava plants are usually free from CBSV infection. Therefore, careful observation on symptoms should be made when diagnosing for CBSD and whenever possible, the symptomatology should be supplemented with molecular detection of the virus.

The relative distribution of CBSV in various organs of the infected plants was also established. This knowledge will guide the choice of the most appropriate sample for CBSV detection. Although CBSV was detected in most of tested tissues, the non-necrotic root tissue, young tender leaves (recently formed) and the youngest symptomatic leaves were the best samples. The study findings were also similar to those of Maruthi *et al.*, (2005) who did not detect CBSV in seeds obtained from CBSV-infected plants. The virus is not known to be seed-transmitted. This created a fundamental base for establishment of CBSV-free plants regardless of the CBSD status of the seed source plants. This will ease regional and international exchange of

breeder seeds without fear of spreading CBSV. The study established that, disease-free plants cannot be regenerated from CBSD-affected plants.

Different cultivars had varied response to CBSD across seasons. This was consistent with earlier reports (Jennings, 1957; Storey and Doughty, 1951) that the disease severity in CBSD-affected plants depends on the cultivar characteristics. CBSD foliage symptoms were more common than the stem symptoms. The foliage and stem severity of the disease were noted to increase with plant age and highest disease incidences and severities were recorded after 9 months in all the tested cultivars. Some cultivars sustain dual infection from CBSV and CMVs. However, there is no correlation between CBSD disease indices and CMD severity. The rate of symptoms expression was also independent of the duality of the infection from the two diseases but the foliage CBSD symptoms were often obscured posing difficulties in diagnosis. CBSD incidence was likewise not related to the population of adult *B. tabaci*. The environmental temperature (Jennings, 1957; Nichols, 1950a) was reported previously to affect disease severity in CBSD-affected plants. The current study established that the critical temperature which is lethal to CBSD is 26 ^{0}C day and 18 ^{0}C night temperatures, respectively. At this temperature, the disease became more severe and all CBSD-affected plants died within 50 days after exposure. Quick expansion of foliage vein chloroses from tertiary to secondary and primary veins was noted. The rapid increase in the number and size of chlorotic blotches was also recorded. Latently-infected plants that had not developed CBSD symptoms for 18 months, exhibited symptoms within 40 days after exposure to this temperature. Reduced development of new leaves was also observed. The study established the critical ranges of temperature that are lethal to CBSD affected plants and confirmed Nichols, (1950a) suspicion of temperature influence on

CBSD. The response of CBSD-affected plants to moisture stress had never been determined despite the suspicion of Nichols, (1950b). For the first time this study established the effect of moisture stress on the diseased plants. CBSD-affected plants of the same cultivar, Albert, responded differently to varied amount of water application. Under similar condition of moisture stress, diseased plants lose water and wither faster than CBSV-free plants. The effect can be lethal in CBSD-affected plants while healthy ones are virtually unaffected. This suggests that severe damage and high yield losses should be expected in CBSD-affected plants subject to prolonged dry spell.

In conclusion, the current study established vital concepts underlying the epidemiological aspects of CBSD. The disease was found omnipresent throughout the cassava growing areas in Tanzania. The altitude of the respective location had no influence on the distribution of CBSD. CBSV isolates were diverse although the majority claded together. CBSV may be transmitted through cutting tools and leaf harvesting. Like most potyviruses, CBSV is not transmitted through seeds or infected plant debris. In transmission studies, quick results are attainable when CBSV-free scion is grafted on the infected root stock. The major foliage symptoms for CBSD were chlorotic blotches, chlorotic spots and veinal chlorosis. Some CBSD-affected plants exhibited necrotic sepia mass impregnated in the base of leaf petiole. In some instances, senesced leaves did not detach from stems. Poor leaf retention and irregular senescence were also observed. Some foliage symptoms on field plants resembled those of CBSD although they were not necessarily caused by CBSV-infection. Not all apparently symptomless cassava plants are free from CBSV. The virus may be detected from fruits, flowers, leaf buds, young and mature leaves, peelings from tender green portion of the stem and the non-necrotic tissue of the root

cortex. CBSV-free plants canot be generated from diseased plants. The severity of the disease depends on the cultivar characteristics, the duration of infection, the prevailing environmental temperature and the moisture availability. The findings from this study, have contributed appreciably to the understanding of CBSD epidemiology.

6. 2 RECOMMENDATIONS

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

1. CBSD is no longer limited to low altitudes, therefore future surveillance or diagnostic studies should cover all locations where cassava is grown regardless of the altitude.

2. Symptoms-based diagnosis of CBSD should always be supplemented with molecular detection of CBSV.

3. Young tender leaves, youngest symptomatic leaves and non-necrotic portions of the root cortex, presents good samples for the detection of CBSV.

4. In order to minimize the dissemination of CBSV through cuttings, the plants to be used as source of planting materials should selected based on symptoms and tagged earlier while the crop is still actively growing.

5. Cassava breeder seeds may be exchanged regionally and internationally without risk of CBSV spread through infected seeds. Unlike the currently recommended tissue culture technique, the seeds-based exchange of breeding materials is less costly and does not require sophisticated equipments and skilled labour.

6. Preliminary screening of cassava materials suspected to be CBSV-infected should be done at locations with cool temperatures (26 ^{0}C day and 18 ^{0}C night temperatures), particularly when massive screening of breeder material is required.

7. Concerted efforts through breeding for resistance, plant health testing of planting materials and education to farmers are required, to minimize damages caused by CBSD on on-farm cassava.

6.3 AREAS FOR FURTHER RESEARCH

The findings from this study gave insights to areas that require further research including the following:

- The need to explore the molecular and biological characteristics of different CBSV isolates and their effects on infected plants.

- The sequencing of CBSV full genome would provide insights to its genetic constituents and lead to engineering for resistance to the virus.

- Quantification studies to determine whether virus titre changes in plant tissues with age of the plant and/or seasons.

- The relationship between symptoms severity and virus titre in plant tissues needs to be established. This will help understand whether the absence or mild expression of CBSD symptoms in some cultivars, is a mechanism of resistance through restricted multiplication of the virus.

- Other mechanisms of CBSV transmission needs to be explored. This is based on the fact that, the observed transmission efficiency by the tested methods was too low to cause the high CBSD incidence levels reported on exposing originally un-infected plants.

- The nature of interaction between CBSV and Cassava mosaic viruses in dual infected plants and their effects needs to be established.

- The mechanism of susceptibility of CBSD-affected plants to moisture stress was not clearly established. Whether CBSD triggers plants' vulnerability to excessive evapo-transpiration or reduced water uptake from the soil.

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APPENDICES

Appendix i

Table E1. Alignment of partial CP gene nucleotide sequences for the CBSV isolates and other Potyviruses used in this study.

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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
              5         15         25         35         45         55         65         75         85
AF311052  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
AF311053  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
AY008440  AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687174  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
AY008441  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCTGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAGG-
AY007597  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAGG-
AY008442  AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687189  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCCGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687187  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687190  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687188  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687204  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687199  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687198  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687203  AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687200  AAAGCTTTTG TRRTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687183  AAAGCTTTTG TAGTAAAAGC AGCTCGAAAT GCTGGGTACA CAAGCATCGA GAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687185  AAAGCTTTTG TAGTAAAAGC AGCTCGAAAT GCTGGGTACA CAAGCATCGA GAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687193  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCTGGGTATA CAAGCATTGA AAACAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687195  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCTGGGTATA CAAGCATTGA AAACAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687194  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGTATCGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687180  AAAGCTTTTG TAATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGTATCGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
CBSVKE    AAAGCTTTTG TAATAAAAGC AGCTCGAAAT GCCGGGTATA CAAGTATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
FJ687201  AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA GAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687202  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA GAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687168  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687169  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687167  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687175  AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
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FJ687166 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687171 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687170 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687172 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687173 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687176 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687197 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687181 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687186 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687191 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687196 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687178 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCTGGGTACA CAAGCATTGA GAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687179 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCTGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687164 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687182 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687184 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687165 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687192 AAAGCTTTTG TGATAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATAAGT-G GTTAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687206 AAAGCTTTTG TGGTAAAAGC AGCCCGAAAT GCCGGGTACA CAAGCATTGA AAATATTT-G GTGAGGTATT GACTTCCT-- -AGCCGAAG-
FJ687205 AAAGCTTTTG TAGTAAAAGC AGCCCGAAAT GCCGGGTATA CAAGCATTGA AAATAAGT-G GTTGGGTATT GACTTCCT-- -AGCCGAAG-
DQ837303 AAGGCGTTTG TGATAAAAGC TGCTAGAAAT GCTGGATACA CAAGCATTGA AAATAAAT-G GCTAGGTATA GATTTTCT-- -TGCCGAGG-
DQ837304 AAGGCGTTTG TGATAAAAGC TGCTAGAAAT GCTGGATACA CAAGCATTGA AAATAAAT-G GCTAGGTATA GATTTTCT-- -TGCCGAGG-
DQ837302 AAGGCGTTTG TGATAAAAGC TGCTAGAAAT GCTGGATACA CAAGCATTGA AAATAAAT-G GCTAGGTATA GATTTTCT-- -TGCCGAGG-
FJ687177 AAGGCATTTG TGATAAAAGC TGCTAGAAAT GCTGGATACA CAAGTATTGA AAATAAAT-G GTTAGGTATA GATTTTCT-- -TGCCGAAG-
SPMMV    AAACCTTTCA CAGTGAAAGG TGCCGTGACT GCTGGGTATG CTAATGTTCA GGATGCTT-G GCTAGGTATT GACTTTCTTC GAGACACGA-
CVYV     AAAGCATTCA CTGTGAAGGC TGCCCGTAAC GCTGGGTACA CTGAGATTGA GGATCAGT-G GCTTGGAATT GATTTTCT-- -GGCTGAAG-
TEV      CGACCATATA TGCCTAGGTA TGGTCTACAG AGAAACATTA CAGACAT--G AGTTTGTCAC GCTATGCGTT CGACTTCTAT GAACTAACTT

         ....|....| ....|....| ....|....| ....|....| ....|....| ...
             95        105        115        125        135
AF311052 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
AF311053 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
AY008440 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687174 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
AY008441 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAGA TTTTGGCCGC TAA
AY007597 CACAATTGTC ACAAA-GCCA GCTTGATATC AAACATCAAA TATTGGCTGC CAA
AY008442 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687189 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687187 CACAATTGTC ACAATTGCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
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FJ687190 CACAATTGTC ACAATTGCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687188 CACAATTGTC ACAAT-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687204 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687199 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687198 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687203 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687200 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
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FJ687185 CACAATTGTC ACAAA-GCCA ACTKGATATC AAACATCAAA TATTGGCTGC TAA
FJ687193 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC CAA
FJ687195 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC CAA
FJ687194 CACAATTGTC ACAAA-GCCA GCTGGATATC AAACATCAAA TATTGGCTGC TAA
FJ687180 CACAATTGTC ACAAA-GCCA GCTGGATATC AAACATCAAA TATTGGCTGC TAA
CBSVKE   CACAATTGTC ACAAA-GCCA ACTGGATATC AAACATCAAA TATTGGCTGC TAA
FJ687201 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687202 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687168 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687169 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687167 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687175 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687166 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687171 CACAATTGTC ACAATTGCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687170 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687172 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
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FJ687197 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
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GRL01408 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
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FJ687196 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
FJ687178 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687179 CACAATTGTC ACAAA-GCCA ACTTGATGTT AAACATCAAA TACTGGCTGC TAA
FJ687164 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687182 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687184 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
FJ687165 CACAATTGTC ACAAT-GCCA ACTKGATATC AAACATCAAA TATTGGCTGC TAA
FJ687192 CACAATTGTC ACAAA-GCCA ACTTGATRTC AAACATCAAA TAYTGGCTGC TAA
FJ687206 CACAATTGTC ACAAA-GCCA ACTTGATATT AAACATCAAA TATTGGCTGC TAA
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FJ687205 CACAATTGTC ACAAA-GCCA ACTTGATATC AAACATCAAA TATTGGCTGC TAA
DQ837303 CTCAATTGTC TCAGA-GTCA ACTTGATATC AAGCATCAGA TTCTGGCAGC GAA
DQ837304 CTCAATTGTC TCAGA-GTCA ACTTGATATC AAGCATCAGA TTCTGGCAGC GAA
DQ837302 CTCAATTGTC TCAGA-GTCA ACTTGATATC AAGCATCAGA TTCTGGCAGC GAA
FJ687177 GCCAACTGTC TCAG--GCCA ACTGGATATC AAGCATCAGA TTCTGGCAGC GAA
SPMMV    TGAAGTTAAC AACAA-AGCA GATGGAAGTC AAACACCAAA TCATCGCAGC GAA
CVYV     CACAACTCTC GCGAA-ACCA ATTAAATATA AAGCACCAGA CACTTGCCGC TAA
TEV      CGAAAACACC TGTTAGAGCG AGGGAGGCGC ATATG-CAAA TGAAAGCTGC TGC
```

Appendix ii

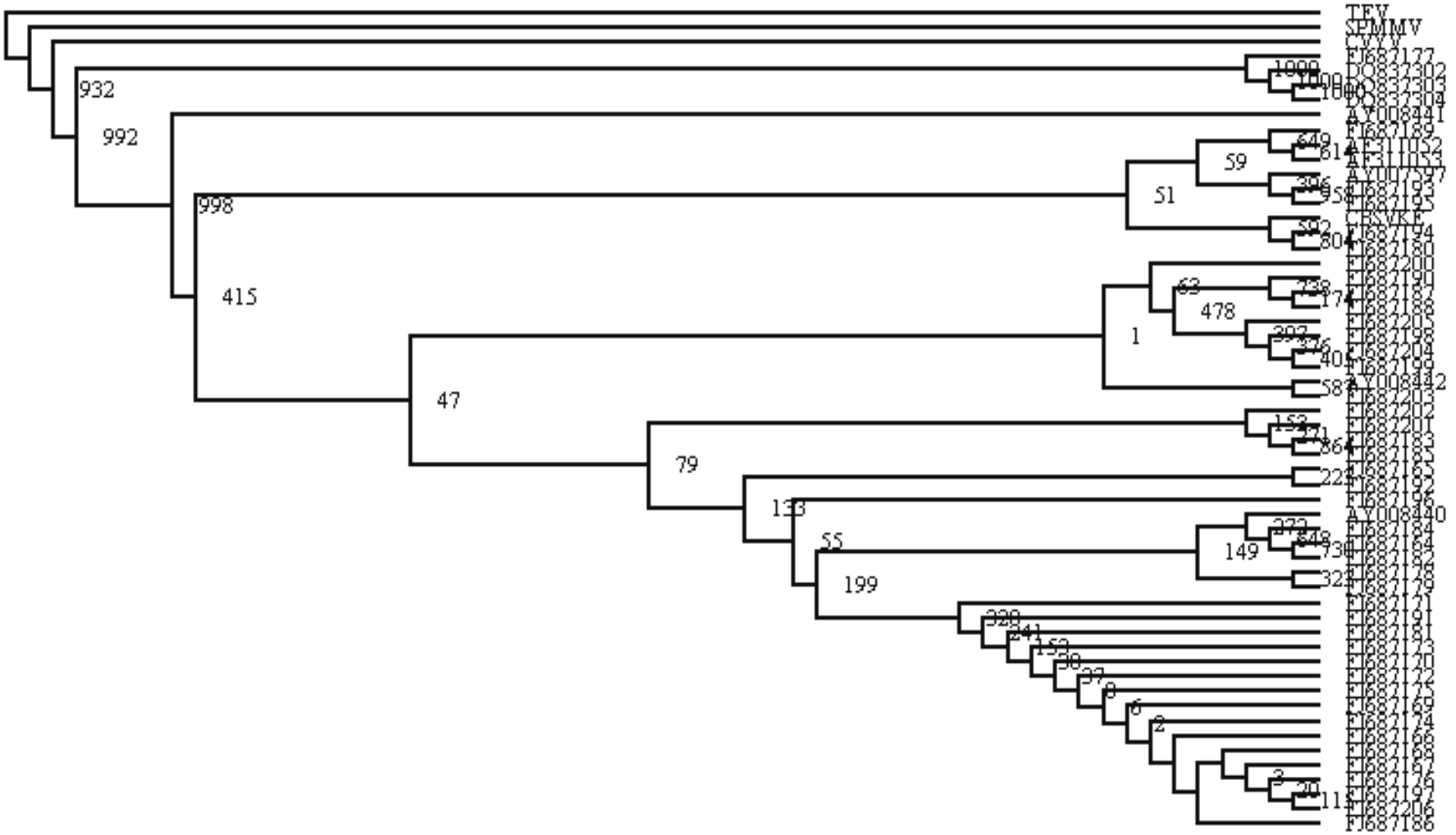


Figure E1. Rooted phylogram for the 53-CBSV-CP nucleotide isolate sequences. CP-gene sequences for TEV, SPMMV and CVYV were included as outliers.

Appendix iii

Table E2. Comparisons of the partial CP nucleotides sequence identities (%) for the new CBSV isolates (this study) and the published CBSV isolates. The new sequences are in the left column and the published ones in the top row. Highly similar sequences (= 80 %) are highlighted (in bold).

Virus isolate	**AF52**	**AF53**	**AY97**	**AY40**	**AY41**	**AY42**	**DQ02**	**DQ03**	**DQ04**	CVYV*	SPMMV*	TEV*
FJ687164	**97.8**	**97.8**	**95.7**	**99.3**	**94.2**	**98.6**	**81.0**	**81.0**	**81.0**	68.6	65.0	14.6
FJ687165	**96.4**	**96.4**	**94.2**	**97.8**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	68.6	64.2	14.6
FJ687166	**96.4**	**96.4**	**94.2**	**99.3**	**92.7**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687167	**96.4**	**96.4**	**94.2**	**99.3**	**92.7**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687168	**96.4**	**96.4**	**94.2**	**99.3**	**92.7**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687169	**96.4**	**96.4**	**94.2**	**99.3**	**92.7**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687170	**96.4**	**96.4**	**94.2**	**99.3**	**92.7**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687171	**95.6**	**95.6**	**93.4**	**98.5**	**92.0**	**96.4**	**80.3**	**80.3**	**80.3**	69.3	64.2	14.6
FJ687172	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687173	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687174	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687175	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687176	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687177	79.4	79.4	77.9	**80.9**	**80.9**	**80.1**	**93.4**	**93.4**	**93.4**	66.9	55.9	11.8
FJ687178	**95.7**	**95.7**	**93.5**	**98.6**	**93.5**	**96.4**	**81.8**	**81.8**	**81.8**	70.8	65.7	14.6
FJ687179	**94.9**	**94.9**	**92.8**	**97.8**	**92.8**	**95.7**	**82.5**	**82.5**	**82.5**	70.1	65.7	14.6
FJ687180	**97.1**	**97.1**	**96.4**	**94.2**	**93.5**	**96.4**	77.4	77.4	77.4	67.2	67.2	14.6
FJ687181	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687206	**94.2**	**94.2**	**92.0**	**97.1**	**90.5**	**94.9**	79.6	79.6	79.6	67.9	65.0	14.6
FJ687182	**97.8**	**97.8**	**95.7**	**99.3**	**94.2**	**98.6**	**81.0**	**81.0**	**81.0**	68.6	65.0	14.6
FJ687183	**94.9**	**94.9**	**92.8**	**93.5**	**92.8**	**94.2**	79.6	79.6	79.6	70.1	65.7	14.6
FJ687184	**97.8**	**97.8**	**95.7**	**99.3**	**94.2**	**98.6**	**81.0**	**81.0**	**81.0**	68.6	65.0	14.6
FJ687185	**94.2**	**94.2**	**92**	**94.2**	**92**	**93.5**	**80.3**	**80.3**	**80.3**	70.1	66.4	15.3
FJ687186	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687187	**98.5**	**98.5**	**96.4**	**95.7**	**94.9**	**97.8**	78.8	78.8	78.8	68.6	65.0	13.9
FJ687188	**98.6**	**98.6**	**96.4**	**95.7**	**94.9**	**97.8**	78.8	78.8	78.8	68.6	65.7	13.9
FJ687189	**99.3**	**99.3**	**97.1**	**96.4**	**95.7**	**98.6**	79.6	79.6	79.6	67.9	65.0	7.3
FJ687190	**98.5**	**98.5**	**96.4**	**95.7**	**94.9**	**97.8**	78.8	78.8	78.8	68.6	65.7	13.9
FJ687191	**95.7**	**95.7**	**93.5**	**98.6**	**92**	**96.4**	**80.3**	**80.3**	**80.3**	69.3	65.0	14.6

FJ687192	**96.4**	**96.4**	**94.2**	**97.8**	**92.8**	**97.1**	**81.8**	**81.8**	**81.8**	68.6	65.0	14.6
FJ687193	**97.8**	**97.8**	**97.1**	**94.9**	**95.7**	**97.1**	**80.3**	**80.3**	**80.3**	67.9	65.7	12.4
FJ687194	**96.4**	**96.4**	**95.7**	**94.9**	**92.8**	**95.7**	78.1	78.1	78.1	67.2	67.9	15.3
FJ687195	**97.8**	**97.8**	**97.1**	**94.9**	**95.7**	**97.1**	**80.3**	**80.3**	**80.3**	67.9	65.7	12.4
FJ687196	**97.1**	**97.1**	**94.9**	**98.6**	**93.5**	**97.8**	**81.8**	**81.8**	**81.8**	70.1	65.7	14.6
FJ687197	**96.4**	**96.4**	**94.2**	**99.3**	**92.8**	**97.1**	**81.0**	**81.0**	**81.0**	70.1	65.0	14.6
FJ687198	**99.3**	**99.3**	**97.1**	**96.4**	**95.7**	**98.6**	79.6	79.6	79.6	69.3	66.4	13.9
FJ687199	**99.3**	**99.3**	**97.1**	**96.4**	**95.7**	**98.6**	79.6	79.6	79.6	69.3	66.4	13.9
FJ687200	**98.6**	**98.6**	**96.4**	**96.4**	**94.9**	**98.6**	79.6	79.6	79.6	68.1	65.0	5.8
FJ687201	**97.1**	**97.1**	**94.9**	**97.1**	**93.5**	**96.4**	**80.3**	**80.3**	**80.3**	70.8	67.2	14.6
FJ687202	**97.1**	**97.1**	**94.9**	**97.1**	**93.5**	**97.8**	**80.3**	**80.3**	**80.3**	70.1	67.2	14.6
FJ687203	**99.3**	**99.3**	**97.1**	**97.8**	**95.7**	**100**	**81.0**	**81.0**	**81.0**	68.6	65.0	13.9
FJ687204	**99.3**	**99.3**	**97.1**	**96.4**	**95.7**	**98.6**	79.6	79.6	79.6	69.3	66.4	13.8
FJ687205	**99.3**	**99.3**	**97.1**	**96.4**	**95.7**	**98.6**	79.6	79.6	79.6	69.3	66.4	13.8
KECBSV	**97.8**	**97.8**	**95.7**	**94.9**	**94.2**	**97.1**	79.6	79.6	79.6	67.9	66.4	8
AF311052	***	**100**	**97.8**	**97.1**	**96.4**	**99.3**	**80.3**	**80.3**	**80.3**	68.6	65.7	7.3
AF311053		***	**97.8**	**97.1**	**96.4**	**99.3**	**80.3**	**80.3**	**80.3**	68.6	64.7	7.2
AY007597			***	**94.9**	**95.7**	**97.1**	**80.3**	**80.3**	**80.3**	66.4	67.2	7.3
AY008440				***	**93.5**	**97.8**	**81.8**	**81.8**	**81.8**	70.8	64.2	14.6
AY008441					***	**95.7**	**83.2**	**83.2**	**83.2**	69.3	66.4	7.3
AY008442						***	**81.0**	**81.0**	**81.0**	70.1	65.0	13.9
DQ837302							***	**100**	**100**	62.0	59.1	12.4
DQ837303								***	**100**	62.0	59.1	12.4
DQ837304									***	62.0	59.1	12.4
CVYV*										***	41.6	6.6
SPMMV*											***	9.3
TEV*												***

*Selected Ipomoviruses (CVYV and SPMMV) and a *Potyvirus* (TEV) whose sequences were included for compa

*** Point of intersection at which comparison of the same sequences led to the same outcome.

Appendix iv

Table E3. Comparisons of the partial CP gene amino acids sequence identities (%) for the new CBSV isolates (this study) and the published CBSV isolates. The new sequences are in the left column and the published ones in the top row. Highly similar sequences (= 80 %) are highlighted (in bold).

Virus isolates	**AF52**	**AF53**	**AY97**	**AY40**	**AY41**	**AY42**	**DQ02**	**DQ03**	**DQ04**	CVYV*	SPMMV*	TEV*
FJ687164	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687165	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**80.0**	48.9	11.1
FJ687166	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687167	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687168	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687169	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687170	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687171	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**82.2**	51.1	11.1
FJ687172	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687173	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687174	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687175	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687176	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687177	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	77.8	48.9	6.7
FJ687178	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687179	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	77.8	51.1	11.1
FJ687180	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687181	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687206	**93.3**	**93.3**	**93.3**	**93.3**	**93.3**	**93.3**	**93.3**	**93.3**	**93.3**	**80.0**	48.9	11.1
FJ687182	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687183	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687184	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687185	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687186	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687187	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**82.2**	51.1	11.1
FJ687188	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**82.2**	51.1	11.1

FJ687189	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	77.8	46.7	11.1
FJ687190	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**82.2**	51.1	11.1
FJ687191	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**95.6**	**80.0**	51.1	11.1
FJ687192	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	77.8	48.9	11.1
FJ687193	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687194	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687195	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687196	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687197	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687198	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687199	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687200	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**80.0**	48.9	11.1
FJ687201	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687202	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687203	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
FJ687204	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
FJ687205	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**97.8**	**82.2**	51.1	11.1
KECBSV	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AF311052	***	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AF311053		***	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AY007597			***	**100**	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AY008440				***	**100**	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AY008441					***	**100**	**100**	**100**	**100**	**80.0**	48.9	11.1
AY008442						***	**100**	**100**	**100**	**80.0**	48.9	11.1
DQ837302							***	**100**	**100**	**80.0**	48.9	11.1
DQ837303								***	**100**	**80.0**	48.9	11.1
DQ837304									***	**80.0**	48.9	11.1
CVYV*										***	53.3	11.1
SPMMV*											***	19.6
TEV*												***

*Selected Ipomoviruses (CVYV and SPMMV) and a *Potyvirus* (TEV) whose sequences were included for compa

*** Point of intersection at which comparison of same sequences led to the same outcome.

Appendix v

Table E4. Analysis of variance for CBSD foliage incidence for twelve months in 2006/2007

Source of variation	d.f	Mean square	F.	P-value
Replication	2	965	2.83	
Variety	5	18170	53.33	<0.001
Residual	10	341	4.49	
Time	11	1380	18.20	<0.001
Variety.Time	55	273	3.60	<0.001
Residual	132	76		
Total	215			

Table E5. Analysis of variance for CBSD foliage severity score in 2006/2007

Source of variation	d.f.	Mean square	F.	P-value
Replication	2	1.2	1.80	
Variety	5	24.4	36.14	<0.001
Residual	10	0.7	5.46	
Time	11	1.6	12.74	<0.001
Variety.Time	55	0.3	2.58	<0.001
Residual	132	0.1		
Total	215			

Table E6. Analysis of variance for CBSD stem incidence in 2006/2007

Source of variation	d.f.	Mean square	F.	P-value
Replication	2	70	0.14	
Variety	5	4994	10.27	<0.001
Residual	10	486	4.60	
Time	11	1306	12.34	<0.001
Variety.Time	55	225	2.13	<0.001
Residual	132	106		
Total	215			

Table E7. ANOVA for CBSD stem severity score in 2006/2007

Source of variation	d.f.	Mean square	F	P-value
Replication stratum	2	0.3	0.28	
Variety	5	17.1	14.68	<0.001
Residual	10	1.2	3.53	
Time	11	2.9	8.90	<0.001
Variety * Time	55	0.6	1.96	<0.001
Residual	132	0.3		
Total	215			

Table E8. ANOVA for CBSD foliage incidence in 2007/2008

Source of variation	d.f.	Mean square	F	P-value
Replication	2	965	2.83	
Variety	5	18170	53.33	<0.001
Residual	10	341	4.49	
Time	11	1380	18.20	<0.001
Variety * Time	55	273	3.60	<0.001
Residual	132	76		
Total	215			

Table E9. ANOVA for CBSD foliage severity score in 2007/2008

Source of variation	d.f.	Mean square	F. .	P-value
Replication	2	34.3	216.20	
Variety	5	11.1	69.51	<0.001
Residual	10	0.2	0.22	
Time	11	4.3	6.07	<0.001
Variety * Time	55	0.3	0.37	1.000
Residual	132	0.7		
Total	215			

Table E10. ANOVA for CBSD stem incidence in 2007/2008

Source of variation	d.f.	Mean square	F	P-value
Replication	2	1088	5.47	
Variety	5	4980	25.03	<0.001
Residual	10	199	0.64	
Time	11	167	0.54	0.874
Variety * Time	55	255	0.82	0.794
Residual	132	310		
Total	215			

Table E11. ANOVA for CBSD stem severity score in 2007/2008

Source of variation	d.f.	Mean square	F	P-value
Replication	2	7.2	8.37	
Variety	5	12.6	14.62	<0.001
Residual	10	0.9	1.00	
Time	11	0.9	1.07	0.393
Variety * Time	55	0.8	0.90	0.672
Residual	132	0.9		
Total	215			

HIGHLIGHT OF THE NEW FINDINGS ESTABLISHED IN THIS STUDY

The new findings and developments in CBSD epidemiology that were made during this study include:

1. A robust **guanidium thiocyanate** (GTC) (modified from Monger *et al.,* 2001a) RNA isolation technique. [When compared with other methods used for CBSV-RNA isolation such as CTAB (Lodhi *et al*., 1994) and Pure Link^{TM} Plant RNA purification reagent (Invitrogen), the GTC technique was cheaper in costs of ingredients, quicker and greatly minimized the RNase reaction on the extracted RNA].
2. CBSV was reported for the first time in the Lake and Southern Zones of Tanzania.
3. A total of 43 CBSV isolates from diverse locations in Tanzania were sequenced and data deposited to the public database (NCBI).
4. The study highlighted the possible existence of CBSV strains in Tanzania.
5. New CBSD symptoms were identified and described which may facilitate disease diagnosis.
6. The relationship between symptoms expression and CBSV infections was established.
7. The contribution of cutting tools and leaf harvesting in CBSV transmission and spread were established.
8. The efficiencies of non-vector methods of CBSV transmission were determined.
9. The most suitable samples for CBSV detection by RT-PCR were identified.
10. The effect of temperature on CBSD-affected plants was determined.
11. The response of CBSD-affected plants to moisture stress was also determined.
12. Four papers presented at conferences and five papers to be submitted to peer-reviewed journals.

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Thesis title: Potential invasion areas by the Larger Grain Borer, *Prostephanus truncatus* (Horn) (Col: Bostrichidae) in Zimbabwe with reference to Tanzania; A GIS Based Prediction.

B.Sc. Agronomy, Sokoine University of Agriculture, Tanzania. July 2000.

Special Project Research Title: The Response of Sorghum; *Sorghum bicolor* (L) Moench, to Phosphorus, Nitrogen and Iron applied as nutrients at different rates and combinations to an Oxic Paleustalfs (Eutric Nitosols).

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Recent Publications:

Rwegasira GM, Momanyi G, Kahwa G, Rey MEC, Legg JP and Herron CM, 2008. Widespread occurrence and diversity of *Cassava Brown Streak Virus* (*Potyviridae*; *Ipomovirus*) In Tanzania. *In press*; Phytopathology Journal.

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