# VIRUS-HOST INTERACTIONS IN THE CASSAVA BROWN STREAK DISEASE PATHOSYSTEM

**IBRAHIM UMAR MOHAMMED**

A thesis submitted in partial fulfilment of the requirements of the

Degree of Doctor of Philosophy

Natural Resources Institute (NRI)
University of Greenwich

UK

March 2012

## DECLARATION

I certify that this work has not been accepted in substance for any degree and is not concurrently submitted for any degree other than that of Doctor of Philosophy (PhD) of the University of Greenwich. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

Student: Ibrahim U. Mohammed.....Date.....

Supervisors: Dr. Maruthi M.N. Gowda.....Date.....

Dr. Rory J. Hillocks.....Date.....

To my parents: my father Mohammad Sani Umar who advised that I should go to school at very late age and to the memory of my late mother Zainab Umar Mohammad who assured me that I can do it at that time when I wanted to retreat. May her soul rest in perfect peace and may 'Allah' reward her good deeds with "Jannatul-Firdausi.''

**ACKNOWLEDGEMENTS**

The work reported in this thesis received wide range of support such that it is not possible to mention all the people who were involved on this single page. Nonetheless, I would like to thank especially Dr. Maruthi M.N. Gowda for his supervision, guidance and constant encouragement throughout the study. He made this thesis possible by giving me an ample share of his time and placing no restrictions on his collection of literature, but any error here are solely mine. I am also deeply grateful to Dr. Rory J. Hillocks who generously provided cassava materials, supervision, contribution to my studies and writing up of the thesis especially its presentation. I would like to thank staff and colleagues at Natural Resources Institute (NRI) for their support during this study. I wish to particularly thank Drs Muawiya Musa Abarshi, Peter Wasswa, John Orchard, Mr Mark Parnell and especially Prof Andrew Westby, for their support and encouragement. I thank Drs Carl Collins, Bettina Otto and Sue Seal for their invaluable help, in the glasshouse, insectary and molecular biology laboratory. I also thank Dudley Farman, Charles Whitfield Simon Springate and Mrs Natalie Morley, for technical assistance. I acknowledge the assistance of Dr. Stephen Young, with the statistical analysis (Chapters 4 and 6). I specially thank Elizabeth Roe (Mum) for lending me money to pay for my first year registration at the time when funding from Kebbi State was stopped and for her assistance and support to my family and myself. I would like to thank the Kebbi State Government of Nigeria for funding my studies. My many friends and relatives constantly showered me with love and support in the good and bad times that a Ph.D. inevitably involves. All the above transformed my endeavour into a surmountable enjoyable and worthwhile experience.

Last but not least, I wish to thank my family who, in a very special way, enabled me to realise my dream of completing my studies. To Nafisatu S.T. Sulaiman, my wife, thank you for your support, encouragement and taking care of our teenage children while I was always in the school. To Zainab Ummi Ibrahim, Ahmed Abba Ibrahim, Hussainatu Mami Ibrahim and Mohammad Mahmood Ibrahim, thank you for your support, encouragement and belief that the sky was the limit in your father's pursuit of his studies.

**ABSTRACT**

The research seeks to understand the virus-host plant interactions for cassava brown streak disease (CBSD) caused by two viruses, *Cassava brown streak virus* (CBSV) and *Ugandan Cassava brown streak virus* (UCBSV) of the genus *Ipomovirus*, family *Potyviridae*. The diversity of six CBSD isolates from the endemic (Kenya, Malawi, Mozambique and Tanzania) and the recently developed epidemic areas (Uganda) of the disease in eastern Africa was studied. Five cassava varieties differing in virus resistance levels; Albert, Columbian, Ebwanateraka, TMS60444 (all susceptible) and Kiroba (tolerant) were graft-inoculated with the UCBSV and CBSV isolates. Based on a number of parameters, the isolates can be grouped into two main categories; severe and milder forms. Transmission of viruses using non-vector modes confirmed that CBSV was sap transmissible from cassava to cassava. Graft-inoculation of infected scions onto CBSD-free cassava plants was the most efficient mode of transmission which resulted in 80 and 100% rate for UCBSV and CBSV respectively. The two virus isolates were not transmitted through contaminated tools and hands. The effect of host-tolerance on virus was investigated in a long-term experiment where three cassava varieties Albert, Kiroba and Kaleso (field-resistant to CBSD) were graft-inoculated with UCBSV and CBSV. The three cassava varieties showed differences in virus movement, symptom development, severity and relative virus titres. The mechanisms of resistance to CBSD were investigated by making cuttings, from various parts of the plants, and a greater number of disease-free plants were generated from cuttings made from Kaleso than Kiroba and Albert. The fecundity of *B. tabaci* and its ability to transmit the virus were determined and results indicated no significant differences in the ability of the three cassava varieties to support whitefly development. Finally, thermal and chemical treatments of tissue cultured plants were conducted and the combinations of both treatments produced the greatest number of disease-free plants in all three varieties; Kaleso (50%), Kiroba (44%) and Albert (35%). The information generated in this thesis has greatly improved our understanding of the interactions between the three biotic factors; the host, virus and vector in the CBSD-pathosystem, which would be highly useful in designing effective disease management strategies.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## ABBREVIATIONS

| | |
|---|---|
| ACMV | *African cassava mosaic virus* |
| a.s.l. | Above sea level |
| BAP | Benzlaminopurine |
| Bt | *Bacillus thuringiensis* |
| bp | Base pair |
| CMD | Cassava mosaic disease |
| CMBs | Cassava mosaic begomoviruses |
| CAD | Cassava anthracnose disease |
| CBB | Cassava bacterial blight |
| CBSD | Cassava brown streak disease |
| CBSV | *Cassava brown streak virus* |
| UCBSV | *Ugandan Cassava brown streak virus* |
| CP | Coat protein |
| cDNA | Complementary deoxynucleic acid |
| CVYV | *Cucumber vein yellowing virus* |
| $^{O}$N | Degree north |
| $^{O}$S | Degree south |
| $ | Dollar |
| DRC | Democratic Republic of Congo |
| DB | Die back |
| EACMV | *East African cassava mosaic virus* |
| EAAFRO | East African Agriculture and Forestry Research Organisation |
| EACMV-UG | *East African cassava mosaic virus*-Uganda |
| EDTA | Ethylenediaminetetraacetic Acid |
| ESP | Epidemic Spastic Paraparesis |
| ET | Efficiency of therapy |
| Ha | Hectare |
| CIAT | International centre for tropical agriculture |
| IITA | International institute for tropical agriculture |
| ILTAB | International Laboratory for Tropical Agricultural Biotechnology |
| IRAT | Institute de Recherches Agronomiques Tropicales |
| KARI | Kenya Agricultural Research Institute |

| | |
|---|---|
| Kcal | Kilocalories |
| KDa | Kilodaltons |
| LM | Leaf mottling |
| LC | Leaf collapse |
| LCH | Leaf chlorosis |
| LL | Local lesion |
| Mo | Mosaic |
| M | Marker |
| M. | Manihot |
| Mt | Metric tonnes |
| MS | Murashige and Skoog |
| NRI | Natural Resources Institute |
| N. | Nicotiana |
| NPV | *Nuclear polyhedrosis virus* |
| PCR | Polymerase chain reaction |
| Pr | Percentage response |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcription polymerase chain reaction |
| RT-qPCR | Reverse transcription-quantitative polymerase chain reaction |
| Spp. | Species |
| SPMMV | *Sweet potato mild mottle virus* |
| SDW | Sterile distilled water |
| SG | Stunted growth |
| SSA | Sub-Saharan Africa |
| TMS | Tropical *Manihot selection* |
| TME | Tropical *Manihot esculenta* |
| TC | Tissue culture |
| UK | United Kingdom |
| US | United States of America |
| USD | United States of America dollar |
| VC | Vein clearing |

# CHAPTER 1: General introduction, objectives and experimental plan

## 1.1 Importance of cassava in Africa

Cassava is one of the world's most important food crops (Nassar and Ortiz, 2010; Legg *et al*., 2011) as it is the source of carbohydrate for more than 800 million people in the tropical world (Dixon, *et al.,* 2002) and providing over 500 daily calories for over 100 million people (Chavez *et al*., 2005). Cassava is the third most important source of carbohydrates in Sub-Saharan Africa (SSA) and the most important food crop in Nigeria, superseded only by rice, maize and millet within the tropics (Mbwika, 2002; Nassar, 2002; Herzberg *et al.,* 2004; Devries *et al*., 2011). Cassava generates cash income for a large number of households in comparison with other food staples (Nassar, 2002) making it an essential contributor to food security, poverty alleviation and economic growth in the SSA region (Kawano, 2003). The roots and leaves are available throughout the year (Ntawuruhunga *et al*., 2006), thus cassava is an important food security crop, especially in drought-stricken areas (Chavez *et al*., 2005).

Cassava is the main source of carbohydrates, vitamins and minerals for the many poor in SSA, some parts of East Asia and large parts of Latin America (Salcedo *et al*., 2010). There is an increased need for cassava production in developing nations to meet the demand for cassava as a human food. The search for energy has also stimulated research into cassava as a source of bio-ethanol (Plucknett, 1984). Thus cassava provides a major opportunity to increase foreign exchange earnings for SSA countries. Cassava has several advantages over other food staples including rice, maize, sorghum and millet, especially in areas where there are weak market infrastructures, scanty, uncertain rainfall and poor resource base (Nweke *et al.,* 2002). Food security is the first priority for farming households in Africa. This security however, is being threatened by two important virus diseases: cassava mosaic disease (CMD) and cassava brown streak disease (CBSD).

## 1.2 History and importance of CBSD

CBSD was first described in East Africa by Storey (1936). Its causative pathogen has been confirmed relatively recently as *Cassava brown streak virus* (CBSV) (Monger *et al*., 2001a). CBSD is now known to be caused by two viruses CBSV and *Ugandan Cassava brown streak virus* (UCBSV) of the genus *ipomovirus*, family potyviridae (Alicai *et al*., 2007; Mbanzibwa *et al.,* 2009; Monger *et al*., 2010; Winter *et al.,* 2010; Mbanzibwa *et al.,* 2011). Differences in symptom expression associated with UCBSV and CBSV have been demonstrated using the herbaceous test plant, *Nicotiana benthamiana*, but such differences were less apparent for infections in cassava (Winter *et al*., 2010). The genome structure of CBSV (9069-9070 nt) is longer than that of UCBSV (8995-9008 nt) and both encodes a polyprotein of 2912-2916 and 2901-2902 aa respectively (Mbanzibwa *et al.,* 2011).

CBSD is endemic among the East African coastal cassava growing areas, where it was earlier believed to be restricted to only low and mid altitudes of up to 1000 m above sea level (a.s.l.) (Nichols 1950; Hillocks *et al* 1996). CBSD is now reported in Tanzania, Uganda, Malawi, Kenya, Mozambique, and Zambia (Hillocks, 2003; Hillocks, 2006; Alicai *et al*., 2007; Winter *et al*., 2010). CBSD is a more important cause of crop loss in these regions than was earlier believed (Hillocks and Jennings, 2003) since the disease causes both quantitative and qualitative reduction in total root yield by rotting of roots, rendering them unmarketable and unpalatable. CBSD is thus threatening the livelihood and food securities of millions of producers and cassava consumers in SSA. The rapid spread of CBSD in areas considered previously to be outside the natural range of the disease requires development of control measures that will be appropriate and sustainable for cassava producers.

CBSD can be controlled by cultural practices such as roguing, selecting disease-free planting materials, early harvesting and planting resistant varieties (Hillocks *et al.,* 1996; Hillocks and Jennings, 2003; Kanju *et al*., 2003). As the root symptoms of CBSD usually begin to develop 4-8 months after sprouting, farmers harvest early to avoid the disease. The method of early harvesting before the crop reaches its full potential results in less yields (Hillocks *et al.,* 2002). Therefore,

the best control method for CBSD is the use of tolerant and resistant varieties, since most of the tolerant varieties matured without root symptoms or with only mild root symptoms (Hillocks, 2003; 2006). This would allow cassava to be left in the fields to achieve maximum yield potential and permit staggered harvesting, which would increase overall production and enhance the role of cassava as a famine reserve crop in SSA.

Research has been conducted since the 1930s in an attempt to secure resistance to CBSD (Storey, 1947). However, the mechanisms of resistance/tolerance in cassava to CBSD are still not fully understood. Determining the mechanisms of host-plant resistance to CBSD could be of great practical assistance to cassava breeders as the recent outbreak of CBSD from the endemic areas to high coastal areas of Uganda requires urgent control.

Little information is available on virus-host plant interactions in the CBSD pathosystem. It was unknown if the so called 'resistance' to CBSD is due to a host response mechanism after infection with the virus, or inability of the whiteflies (*Bemisia tabaci*), the vector of UCBSV and CBSV, to transmit the viruses to a particular variety. These were investigated by measuring rate of virus multiplication, virus movement and spread in CBSD-susceptible and -tolerant cassava varieties in long-term experiments. Whether the tolerance/resistance to CBSD is because of the inability of its whitefly vector to feed on tolerant/resistant cassava was also investigated by conducting whitefly fecundity experiments and the rate of UCBSV and CBSV transmission by *B. tabaci* on cassava varieties with different CBSD tolerance levels. Reversion is a characteristic feature of virus-resistant cassava varieties where healthy plants can be obtained from making stem cuttings of the previously diseased plants (Fondong *et al*., 2000). This has been well documented for CMD while no such studies have been conducted on CBSD. Whether or not reversion occurred for CBSD was investigated by making stem cuttings of diseased plants. Attempts were also made to regenerate virus-free cassava plants by eliminating the virus using tissue culture techniques, thermotherapy, chemotherapy and simultaneous application of the three therapies.

### 1.3 Aims and Objectives

The main aim of this study was to achieve an improved understanding of the mechanisms of resistance to CBSD through several host-virus-vector interaction experiments. The research has the following four inter-linked objectives;

**Objective 1**: To determine symptom development and diversity by different CBSD isolates on cassava and herbaceous host plants with the aim of determining whether a severe form of the virus is associated with the recent CBSD outbreak in Uganda.

**Objective 2**: To determine the mode of transmission of CBSV by non-vector methods such as graft and mechanical transmission, using contaminated tools, and cultural practices such as cassava leaf harvesting/picking.

**Objective 3**: Understanding the mechanisms of resistance/tolerance to CBSD in cassava by determining virus-host-vector interactions (rate of virus multiplication, spread and titre) and through reversion experiments.

**Objective 4**: To eliminate virus from CBSD-infected cassava varieties by tissue culture, thermotherapy, and chemotherapy and through the simultaneous application of the most effective therapies.

### 1.4 Experimental plan

**Objective 1**: Determine symptom diversity in CBSD isolates on cassava and herbaceous host plant

Experiment 1*:* Inoculate five susceptible cassava varieties with six CBSV isolates from different countries and compare symptom development and variations.

Experiment 2*:* Inoculate selected herbaceous experimental host plants (*Nicotiana* species) with six virus isolates and compare symptom variations.

**Objective 2**: To determine the non-vector modes of CBSV transmission and their efficiency.

Experiment 1: Inoculate selected susceptible cassava varieties using sap-inoculation, sap-injection, contaminated tools, leaf picking and graft-inoculation.

**Objective 3**: Understanding the mechanisms of resistance/tolerance to CBSD in cassava by determining the virus-host-vector interactions

Experiment 1*:* Examine virus distribution in the host in relation to leaf and root symptoms

Experiment 2*:* Compare varieties with respect to rate of virus spread within the plants

Experiment 3*:* Compare tolerant and susceptible varieties with respect to virus titre

Experiment 4*:* Can virus-free cuttings be obtained from infected plants? – Make cuttings from various parts of the plant both in the susceptible and resistant varieties and study mechanisms of reversion.

Experiment 5*:* Measure the fecundity and survival of whiteflies and the rate of UCBSV and CBSV transmission by *B. tabaci* on susceptible (Albert), tolerant (Kiroba) and resistant (Kaleso) cassava varieties.

**Objective 4**: To eliminate virus from CBSD-infected cassava varieties

Experiment 1*:* Attempts to eradicate virus from the plant by tissue culture techniques, thermotherapy, chemotherapy and simultaneous application of the therapies.

# CHAPTER 2: Literature review

## 2.1. Global cassava production

In recent years global cassava production has shown a tremendous increase and is expected to show continued growth over the coming years, with Africa producing more than half of the global production. Over 234 million tonnes of cassava were produced worldwide in 2009, of which over 119 million tonnes were from Africa (FAOSTAT, 2009). Nigeria is the world's leading cassava producer, generating over 37 million tonnes in 2009 (FAOSTAT, 2009). East African countries produced 27 million tonnes of cassava in 2009 and ranked third in production in Africa (Table 2.1). In most areas where cassava is produced it was believed that increased production is due to increases in area under cultivation rather than yield per hectare (Hillocks, 2002). Cassava is cultivated by planting either stem cuttings or seeds. For cassava plants grown from stem cuttings tuberous roots are formed by secondary thickening of a proportion of the adventitious roots that develop usually at the basal end of the cutting. Plants grown from seed initially form a taproot from which adventitious roots arise later, some of which develop into storage roots (Cooke and Coursey, 1981). Roots of cassava plants are the main storage organ and economic part of the plant and their characteristics differ between varieties (Alves, 2002).

Cassava is one of the simplest crops to produce because propagation by cuttings is relatively easy and most varieties can tolerate poor climatic conditions, pests, diseases and deteriorated soil conditions (Hillocks and Jennings, 2003; Jaramillo *et al.*, 2005). Cassava productivity per unit area per unit time is the greatest when compared to sweet potato (*Ipomoea batatas*), potato (*Solanum tuberosum*), millet (*Pennisetum typhoides* Burm), sorghum (*Sorghum bicolor* L.), maize (*Zea mays* L) and rice (*Oryza sativa)*, (Scott *et al.*, 2000) at 25% more than maize and 40% more than rice (Agwu and Anyaeche, 2007). In areas of high population density, such as southern Malawi, cassava is replacing maize as a primary food crop (FAO, 2010), which may be due to the combined effects of declining soil fertility and climate change.

Table 2.1: Cassava production in the world, Africa and specifically in some East African countries

| Country | Cassava production (tonnes) | Yield (tonne/hectare) |
|---|---|---|
| World | $234.0 \times 10^6$ | 12.4 |
| Africa | $119.0 \times 10^6$ | 9.7 |
| Western Africa | $59.0 \times 10^6$ | 11.7 |
| Central Africa | $33.0 \times 10^6$ | 9.4 |
| East Africa | $27.0 \times 10^6$ | 7.2 |
| Tanzania | $5.9 \times 10^6$ | 5.5 |
| Mozambique | $5.7 \times 10^6$ | 5.3 |
| Uganda | $5.2 \times 10^6$ | 12.6 |
| Madagascar | $2.7 \times 10^6$ | 6.7 |
| Malawi | $3.9 \times 10^6$ | 20.3 |
| Rwanda | $1.0 \times 10^6$ | 7.2 |
| Zambia | $0.9 \times 10^6$ | 4.5 |
| Kenya | $0.8 \times 10^6$ | 11.6 |

Source of data: FAOSTAT (2009)

Increased cassava production in Africa could also be attributed to the rapid population growth and poverty which encouraged subsistence farmers to search for cheaper sources of food energy. Genetic research and better agronomic practices were the two main driving forces that have also contributed to the rapid growth of cassava production in Africa (Nweke *et al.,* 2002).

Cassava is mainly grown for human consumption and provides 60% of the daily energy intake in SSA (Taylor *et al*., 2004). Cassava is grown and consumed by the world's poorest and most food insecure households (Carter *et al*., 1992; Henry and Hershey, 2002) and adopted in most areas where it is now grown in some SSA countries as a famine-reserve crop (Hahn, 1984). Before the introduction of cassava from South America, the traditional staple crops in most cassava producing areas of Africa were sorghum, millet, rice and yam. Cassava's reliability as a source of food and its ability to fill the hungry gap when other food staples are not available, particularly in the time of drought, favoured its cultivation in SSA (Barratt, *et al*., 2007). Further expansion of cassava production in most African countries may have been constrained by the current CMD and

CBSD epidemic occurring in Africa, especially in the non coastal highland areas of East Africa.

### 2.1.1 Cassava origin and distribution in Africa

About 98 species of the wild genus *Manihot* exist in the western hemisphere of which only *Manihot esculenta* does not exist in wild state (Rogers and Appan, 1973). The geographical origin of agricultural domestication of cassava has been disputed for a long time. Archaeological evidence indicates that cassava originated in the South and Central America (Rogers, 1963; Leone, 1977). Wood (1985) suggested Brazil as the place of cassava origin. Portuguese traders first introduced cassava into West Africa between 16$^{th}$ and 18$^{th}$ century in slave ships (Jones, 1959; Nweke *et al*., 2002; Monger *et al*., 2010). The growth of cassava production and distribution in Nigeria and Benin Republic are attributed to the freeing of Brazilian slaves who returned to the area around 1800 (Agboola, 1968). Other attributes possessed by cassava are its low labour requirements during cultivation and flexibility of its harvest period (Rhodes, 1996). Ability to produce a crop in poor soils was thought by earlier researchers to be a reason favouring cassava distribution (Jones, 1959). This was supported by Agboola (1968), who thought increased importance of cassava was associated with declining fallow lengths in the Savannah area of West Africa. The diffusion of cassava into African agriculture was described as 'self–spreading' by Nweke *et al.* (2002). Cassava arrived in East Africa in the 19th century (Jones, 1959). Purseglove (1968) indicates that cassava was taken to East Africa from Brazil in 1736 and was noted in Zanzibar in 1799. The explorer Speke, found no cassava on the western shore of Lake Victoria in 1862, but the crop was recorded in Uganda in 1878 (Hillocks, 2002). In addition, Wood (1985) noted that Mbunda migrants from northeast Angola introduced cassava to the upper Zambezia in the 1830s.

Linguistic studies based on the similarity of local names for cassava identified several routes, which accounted for the distribution of cassava between Central and East Africa (Pasch, 1980). The first route extended from Angola to Mozambique, while a second route led from central Zaire to northern Zimbabwe. A third route connected the Lozi (Zimbabwe borders) to the Tonga in Zambia. In the 1850s, cassava was noted by German travellers in north Cameroon among

Fulani who were probably responsible for the spread of the crop in the area (Ekanayake *et al*., 1997). Moreover, cassava was also thought by earlier scientists to promote laziness, soil depletion and malnutrition (Ross, 1975). This view may likely be due to low labour requirement in its cultivation; its ability to grow well on marginal soil and its low-level protein, vitamin and mineral content. Nevertheless, cassava's special characteristics make it well adapted to farmers' risk bearing strategies and allow it to be grown under a great diversity of circumstances. The crop is now widely grown in tropical and subtropical areas including Africa, South Asia and South America (Hillocks, 2002).

### 2.1.2 Cassava taxonomy

Cassava (*Manihot esculenta* Crantz) belongs to the *Fruiticosae* section of the genus *Manihot* of the dicotyledonous family *Euphorbiaceae (*Table 2.2). Comprising about 7200 species, the *Euphorbiaceae* include several economically important plants such as: rubber tree (*Hevea brasiliensis*), castor oil plant (*Ricinus comunis*), ornamental plants (*Euphorbia* spp) and cassava (Roggers and Appan, 1973). One defining feature of *Euphorbiaceae* is that all members are known to produce latex. The *Fruiticosae* consist of shrubs that are adapted to savannah or desert condition where as the *Arboreae* consist of tree species (Jennings and Iglesias, 2002). Wild and cultivated cassava species so far studied are diploid with a chromosome number of 2n =36 chromosomes that have regular bivalent pairing at meiosis (Nassar, 1995). Although, polyploidy has been reported in some species such as *M. glaziovii*, it has been suggested that *M. esculenta* is likely to have been derived from the subspecies *flabellifolia* rather than from several progenitor species (Jennings, 1976).

Table 2.2: Taxonomy and classification of the cassava plant

| Classification | Taxonomy |
|---|---|
| Class | Dicotyledoneae |
| Sub-class | Archchlamydeae |
| Order | Euphorbiales |
| Family | Euphorbiaceae |
| Sub-family | Manihotae |
| Genus | Manihot |
| Species | *Manihot esculenta* Crantz |

Source: Format adapted from IITA (2005).

### 2.1.3 Cassava utilization

Cassava is a tropical crop grown between 30 $^{o}$N and 30 $^{o}$S; it has numerous traits that confer comparative advantages in marginal environments (Henry and Hershey, 2002). Cassava tubers can be processed into a wide variety of food, animal feeds and industrial products (Taylor *et al.*, 2004). Due to rapid physiological deterioration of cassava, fresh tuberous roots cannot be stored for long. Cassava is therefore mostly processed after harvest in order to increase its storage life and to reduce the level of toxic cyanide (Bokanga and Otoo, 1991). More than 100 million people obtain over 500 kilocalories (Kcal) per day from cassava (Bokanga and Otoo, 1994). An increase in cassava utilization is expected from 173 million tonnes to 275 million tonnes in the period 1993-2020 (Westby, 2002). This could be due to the recent interest in cassava as one of the alternative feedstocks for ethanol production. This was viewed as an opportunity for the African countries to reduce their exposure to disequilibrium in foreign trade balance (Patino, 2007). Cassava roots and chips are the cheapest feedstock for bio-fuel in comparison with other crop sources such as maize, sugarcane and rice (Patino, 2007). Cassava roots give an ethanol yield of up to 16,000 litres per hectare per unit time as compared to sugar-cane 7,200 litres per hectare per unit time and maize 800 litres per hectare per unit time (Kambewa, 2007). In addition, dried cassava flour was reported to give a yield of 500 litres per tonne of bio-fuel (Bamikole, 2007). It is apparent that establishment of cassava based ethanol industries in Africa would create stable market and boost cassava production in the region (Mhone *et al.,* 2007).

Worldwide starch production from cassava has been estimated to be worth around US $20 billions (FAO, 2010). Cassava utilization in Africa for human consumption alone is 88% with 12% of the crop used as animal feeds and starch (Westby, 2002). In spite of Africa being the world's largest provider of cassava outputs, it has the lowest yield per hectare, perhaps because of low utilization of the crop for purposes other than subsistence (Bokanga, 2007).

Human diseases have been associated with cassava consumption in areas where it is staple food (Westby, 2002; Nzwalo and Cliff, 2011). Cassava contains a potential goitrogenic agent that may aggravate iodine deficiency disorders causing goiter and cretinism, a severe form of mental retardation (Jose and Dorea, 2004). Cassava consumption may result in cyanide exposure if cyanogenic glucosides and their breakdown products are not sufficiently removed from the roots during processing. Dietary cyanide is converted to thiocyanate in the human body. Thiocyanate mimics those of iodine deficiency (Bokanga *et al.,* 1994). However, the goitrogenic action of cassava depends on the glucoside levels in fresh roots, the effectiveness of processing, the frequency of cassava consumption and the iodine intake (Jose and Dorea, 2004). Cretinism and epidemic spastic paraparesis ESP, related to cassava consumption have been reported from Tanzania (Mlingi *et al*., 2011), Mozambique (Ernesto *et al*., 2002; Cliff *et al*., 2011), Zaire (Chabwine *et al*., 2011) and several other countries.

### 2.1.4 Cassava production constraints

The low rate of seed multiplication limits cassava production. A mother plant of cassava produces a maximum of 30 stem cuttings at maturity, whereas in true seed propagation such as millet a single plant can produce hundreds of seeds (Leihner 2002). In most cassava producing areas the yield is far below the potential (Nweke, *et al.,* 1994; Hillocks, 2002), maybe due to several factors such as poor yielding varieties, poor quality planting materials, poor agronomic practices, unavailability of labour, decline in soil fertility as well as the pest and disease incidence. Cassava suffers from many pests and diseases, which can affect the quality and quantity of planting material. A number of diseases are commonly found on cassava throughout the growing season (Harrison *et al.,* 1995). Important diseases of cassava include CMD, CBSD, cassava anthracnose

disease (CAD) (*Collectotrichum gloeosporioides* Penz) (Neunschwander *et al.,* 1987), cassava bacterial blight (CBB) (*Xanthomonas axonopodis* Bondar) and cassava root rot (Munga and Thresh, 2002). Several viruses have been isolated from cassava in SSA, Asia and Americas (Calvert and Thresh, 2002). Of these, CMD and CBSD are the worst. CBSD and CMD pandemics are the result of new encounter situation between host and pathogen (Legg *et al*., 2011). CMD occurs wherever cassava is grown in SSA. Several different geminiviruses including various forms of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) have been reported to be responsible for CMD epidemics (Fauquet and Fargette, 1990; Thresh *et al*., 1998).

The earliest epidemic of CMD occurred in Uganda, in the 1930s and 1940s in which CMD-resistant varieties and phytosanatary measures were used to control the disease for several decades (Jameson, 1964). Until the late 1980s when a major epidemic of a severe form of CMD was reported in north-central Uganda (Otim-Nape *et al*., 1994), the disease had for a long time remained endemic in the country (Thresh *et al*., 1998). The situation with CMD in Uganda then changed to be characterised by the very severe form CMD symptoms which also coincided with an upsurge in whitefly populations (Gibson *et al*., 1996; Otim-Nape *et al*., 1997). This had devastated cassava production in the area and led to the almost complete elimination of the most susceptible cassava varieties (Legg *et al*., 2011). During the early 1990s, many cassava fields were abandoned and widespread food scarcity and some hunger-related deaths were reported in Uganda (Thresh *et al*., 1994). Spread of CMD to the neighbouring countries of the Great Lakes region and beyond was reported (Gibson, 1996), resulting in the classification of the overall occurences as a pandemic (Otim-Nape *et al*., 1997). Molecular characterization of the viruses occurring in the area indicated presence of recombinant CMG variant, EACMV-UG (Zhou *et al*., 1997), as well as occurrence of mixed infections of EACMV-UG and ACMV (Pita *et al*., 2001). The CMD pandemic expanded across many countries in 2005 and annual losses due to CMD in Africa were estimated to be greater than 13 million tonnes (Legg *et al*., 2011). Most recent pandemics are from Angola (Lava Kumar *et al*., 2009) and Cameroon (Akinbade *et al*., 2010).

CMD has been the most researched of all cassava virus diseases, since the breeding of resistant varieties at the Amani research station in the colonial Tangayika in the 1930s. Plants infected with CMD are not killed but their leaves are distorted, root size and number are reduced and stem diameter is also reduced (Otim-Nape, 1990; Owor *et al*., 2005). Yield reduction maybe severe and losses of up to 82% have been reported especially in cassava plants dually infected with ACMV and EACMV forms (Owor *et al.,* 2005). CBSD was considered to be more damaging than CMD in the coastal areas of East Africa with recorded incidences of up to 100% (Hillocks *et al*., 2001, 2002). However, until recently little importance was given to CBSD (Nweke *et al*., 2002), which is currently the major threat to cassava productivity throughout East Africa, Malawi and northern Mozambique.

### 2.2. Cassava brown streak disease occurrence and distribution

In his earlier work on CMD in the season of 1935, Storey recognized the appearance of another virus disease, which he believed to be different from CMD due to leaf mottling (Storey, 1936). While CMD chlorosis is present on young leaves as they unfold, the young leaves in this new disease were normal and only developed the mottle after ageing (Nichols, 1950). This new disease was CBSD and the name derives from the production of dark brown stripes on the otherwise green stem, which are not necessarily the most obvious visible characteristic features of the disease (Hillocks *et al.*, 1996). Hillocks and Jennings (2003) reviewed in detail the distribution of CBSD. Storey (1939) reported that CBSD was widespread in Tanzania, at smaller altitudes only, but was absent at elevations above 1000 m a.s.l. However the disease was later reported at an elevation of 1200 m a.s.l., but these are thought to be due to the movement of infected cassava cuttings from the coast, as whitefly vectors for CBSV (Hillocks and Jennings, 2003; Maruthi *et al*., 2005), used not to be favoured at such high elevations. CBSD was earlier reported as endemic in all coastal cassava-growing regions of East Africa, from Tanzania extending to the north in Kenya and south in Mozambique (Nichols, 1950). Isolated incidences from several surveys (Bock, 1994; Hillocks *et al*., 1996, 1999; Legg and Raya, 1998; Mtunda *et al*., 2003; Gondwe *et al*., 2003; Alicai *et al.,* 2007) confirmed the findings of Nichols (1950). In 1987, cassava fields were severely affected by CBSD between Kibaha

and Morogoro in Tanzania (Thresh, 2003). This finding led to renewed interest to CBSD and root crop researchers called for concerted effort to control the disease (Hillocks and Jennings, 2003).

Until the 1990s, earlier reports on the CBSD incidences were descriptive (Storey, 1939; Nichols, 1950; Bock, 1994). The first quantitative data on CBSD incidences was reported in Tanzania, where the incidence ranged from 19 to 36% in three coastal regions and the southeast region of Mtwara (Legg and Raya 1998). Another more detailed survey conducted in southern Tanzania confirmed greater incidences of CBSD reaching 50% in some fields that are situated close to the coast (Hillocks *et al*., 1999; Muhanna and Mtunda, 2003). Nichols (1950) had also over 60 years ago reported CBSD in Nyasaland now Malawi. Rossel and Thresh again confirmed the presence of the disease in 1993 (Sauti and Chipungu, 1993). An extensive survey undertaken throughout Malawi in July and September 2001 showed that the disease was present at incidences above 75% in many fields along the lakeshore, and those incidences were greater than common at similar altitudes above 600 m in Tanzania (Hillocks and Jennings, 2003). CBSD was reported to be widespread at lower altitudes in the Southern province of Malawi, particularly towards the border with Mozambique, which led Nichols (1950) to conclude that the disease must occur also in Mozambique. However it was not until 1999 that the disease was first reported in Mozambique (Hillocks *et al.,* 2002). Extensive surveys carried out in 1999 confirmed the occurrence of CBSD at high incidences in the Nampula and Zambezia province of Mozambique; these are the major cassava growing areas of the country (Calvert and Thresh, 2002). The overall incidences of CBSD in these areas were 31% in Nampula and 43% in Zambezia (Thresh and Hillocks, 2003). In the coastal areas of northern Mozambique, very high incidences of up to 90 to 100% have been reported (Hillocks *et al*., 2002; Thresh and Hillocks, 2003).

Nichols (1950) reported further observations of CBSD in Uganda at both Serere and Kaberamaido in the north-eastern part of the country. Since then, reports of CBSD in Uganda have been rare and unconfirmed until November 2004, when leaf symptoms typical of CBSD, were observed at Mukono in central Uganda (Alicai *et al.,* 2007). This confirms the re-emergence of CBSD in Uganda 74

years after it was first observed in the 1930s in cassava introduced from Tanzania and controlled by eradication (Jameson, 1964). In Kenya, CBSD was said to be confined largely to the coast and widely distributed (Munga and Thresh, 2002) and the reported incidences of the disease were contradictory. Bock (1994) reported that the disease incidence was low in Kenya, but Munga and Thresh (2002) reported high incidences of 30 to 60%. Among the relatively few records of CBSD occurrences in Kenya, several cases were said to relate to varieties, mostly contained in the National collection (KARI, 1983). In 1999, a molecular diagnostic survey for viruses infecting cassava was conducted in all cassava-growing regions of Kenya and identified the presence of CBSV in most of the samples tested (Were *et al*., 2004). In addition, a significant outbreak of CBSD has been reported from a large multiplication site in the Yala swamp area of western province of Kenya (Ntawuruhunga and Legg, 2007).

Calvert and Thresh (2002) reported a likely movement of cassava planting material across the border into Zimbabwe and Zambia where CBSD is known to occur. Until recently the disease had not been reported in Angola or any of the West and Central African countries. The first report of CBSD symptoms in Angola was in 2005 when Mutunda *et al.* (2003), noted the disease on the local variety 'Rosa', introduced into central Angola from Vigre, a town in northern Angola which borders DRC (Mahungu *et al.,* 2003). This may partially explain the disease spread in Angola, as CBSD was already reported in DRC (Mahungu *et al.,* 2003). With recognition of the threat posed by CBSD to food security in South, East and Central Africa control of the disease has become a priority for research.

### 2.2.1 The viruses infecting cassava in Africa

Sixteen different viruses have been isolated from cassava of which nine were from Africa (Calvert and Thresh, 2002) and these belong to at least four families and genera, namely; *Comoviridae*: *Nepovirus*, *Geminiviridae*: *Begomovirus*, *Potyviridae*: *Ipomovirus*, and *Caulimoviridae*: *Caulimovirus* (Legg and Thresh, 2003). Only two genera are of economic importance in Africa with regard to cassava, namely *Ipomovirus*: UCBSV and CBSV of the family *Potyviridae* and *Begomovirus*: CMBs of the family *Geminiviridae*.

**CMBs**: CMD has been assumed to be caused by a virus for many years (Zimmermann, 1906). Storey and Nichols (1938) provided the first epidemiological information of the virus and further grouped virus strains based on disease severity, into mild and severe forms. Storey and Nichols (1938) further described the mechanism of transmission and concluded that the whitefly ***B. tabaci*** was the vector. However, CMD etiology was not clear until in the late 1970s when Bock and Guthrie (1978) described a virus that could be transmitted by sap inoculation from CMD-infected cassava to *Nicotiana clevelandii* and they named the casual agent of CMD as *Cassava latent virus* (CLV). Again Bock and Woods (1983) determined the etiology of the virus and named it ACMV.

Serological methods with a panel of 17 antibodies (MAbs) to ACMV were used on *Geminiviruses* to determine the epitope profiles of a number of geminivirus strains from cassava and considerable differences were identified (Hong *et al*., 1993). ACMV reacted with 15 monoclonal antibodies and was found in Burundi, Kenya, Uganda, Cameroon, Chad and South Africa (Swanson and Harrison, 1994) while EACMV reacted with nine monoclonal antibodies and was found in Madagascar, Malawi, Zimbabwe, Kenya and Tanzania (Swanson and Harrison, 1994). EACMV was also reported in Cameroon (Fondong *et al*., 2000), where it was previously thought not to occur. *Indian cassava mosaic virus* occurred in India and Sri Lanka, and reacted with only three monoclonal antibodies (Swanson and Harrison, 1994).

Further, molecular approaches to the study of CMBs has led to identification of more viruses such as *South African cassava mosaic virus* (SACMV), (Berrie *et al*., 1998), the Uganda variant of EACMV known as EACMV-UG, which is a recombinant virus with most of the coat protein gene of ACMV inserted in an EACMV-like DNA-A component (Zhou *et al*., 1997). EACMV-UG variants have been isolated in Uganda and were described EACMV-UG1, EACMV-UG2 and EACMV-UG3 (Pita *et al*., 2001). Other examples of recombination in CMBs include; *East African cassava mosaic Zanzibar virus* (EACMZV) (Maruthi *et al*., 2002), and *East African cassava mosaic Malawi virus* (EACMMV) (Zhou *et al*., 1998). Although CMBs are important in all cassava growing regions of Africa, CBSV is now the most economically important virus of cassava in East Africa.

**CBSVs**: Despite Storey's (1936; 1939) assumption that the infectious agent of CBSD is likely to be a virus, there has been some uncertainty in the past over the virus responsible for the disease. Storey's speculation was supported by Kitajima and Costa, (1964) who described elongate virus-like particles that were detected in CBSD-infected samples using an electron microscope. Lennon *et al.* (1986) reported the isolation of elongate filamentous particles 650-690 nm long (Figure 2.1a) from CBSD-infected samples of *N. benthamiana* and concluded that CBSD-infected plants were infected with a novel virus or a complex of two dissimilar viruses. However, Karamagioli (1994) disagreed with Lennon *et al.* (1986) opinions because results from the reverse transcription polymerase chain reaction (RT-PCR) using primers specific to *Carlavirus* and *Potyvirus*, failed to produce amplified products from cassava leaves infected with CBSD.

The particle length of 650 nm was morphologically similar to carlaviruses, hence the suggestion that CBSV belonged to the genus *Carlavirus*. Further work on affected plants led to more conflicting conclusion that CBSD is caused by two virus complex of a *Carlavirus* and a *Potyvirus* (Brunt *et al*., 1996). Again western analysis with an antiserum using *Cowpea mild mottle virus* and CBSV material was reported to have confirmed a serological relationship between these viruses (Brunt, 1996). This caused confusion in assigning the actual genus and family to which CBSV belongs. A more advanced work by Harrison *et al.,* (1995), later highlighted the presence of 'pin-wheel' inclusions typical of potyviruses in CBSD-affected plants. The result of this finding that potyviruses could be involved due to pin-wheel inclusions was later supported by Lecoq *et al*. (2000).
The molecular approach to the study of CBSV begun with partial virus purification from CBSD-infected cassava material collected from Tanzania (Monger *et al*., 2001a). Total RNA was extracted from these purifications and converted to double-stranded cDNA, which were amplified using the polymerase chain reaction (PCR) (Legg and Thresh, 2003). The 3′ terminal region of the genome of CBSV was sequenced, including the coat protein (CP) (Monger *et al.*, 2001b). Findings of this experiment identified CBSV as a member of the genus *Ipomovirus* and provided no evidence that a *Carlavirus* was involved. 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 full genome size of CBSV is reportedly 9,100 bp (Mbanzibwa *et al.,* 2009; Winter *et al.*, 2010). In comparison the partial CP sequences of CBSV revealed close identity with SPMMV in which the genome size is 10,800 bp (Colinet *et al.,* 1996; 1998), CVYV, with genome size as 9,700 bp (Lecoq *et al.,* 2000; Janssen *et al.,* 2005) and SqVYV, with genome size as 9,800 bp (Weimin *et al*., 2008; Li *et al*., 2008). Recent studies have confirmed the occurrence of a new viral species of the virus which was detected in higher altitude areas in Uganda (Alicai *et al*., 2007; Mbanzibwa *et al.,* 2009 Monger *et al*., 2010; Winter *et al*., 2010), which is now referred to as *Ugandan cassava brown streak virus* (UCBSV) (ICTV, 2010).

The unique features of both CBSV and UCBSV are; (a) they both contain a single-stranded (+) ssRNA genome structure, (b) one of the proteins (HAMlh) they encoded is homologous, (c) they both contain a single P1 proteinase and (d) are both lacking the helper component proteinase (HCpro) at the N-proximal part of the poly-protein (Mbanzibwa *et al.,* 2009; ICTV, 2010). The differences between CBSD-associated viruses are found only in the sizes of their genome and poly-protein structures (Mbanzibwa *et al.,* 2009; Winter *et al*., 2010). The genome structure of CBSV (9069-9070 nt) is longer than that of UCBSV (8995-9008 nt) and both encodes a polyprotein of 2912-2916 and 2901-2902 aa respectively (Mbanzibwa *et al.,* 2011). The current view on CBSV and UCBSV genome (Figure 2.1b) (Mbanzibwa *et al.,* 2009; Winter *et al.*, 2010) suggests a deviation from the earlier report that the genome structure for *Potyviridae* is conserved throughout the family (Adams, 2008). Deviation from the viral *Potyviridae* genome has also been reported in other ipomoviruses such as CVYV (Lecoq *et al.,* 2000) and SqVYV (Weimin *et al*., 2008).

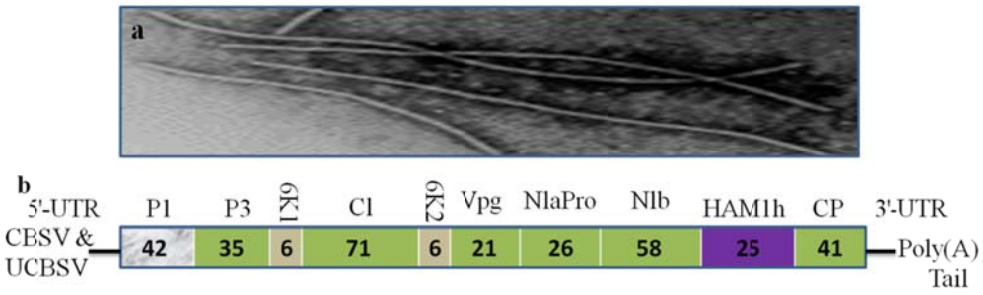



**Figure 2.1**: Particle morphology of CBSV and UCBSV (a) adopted from Lennon *et al.* (1986) and their genome structure (b) format adopted from ICTV (2010). The boxes separated by lines indicate the putative cleavage sites of the polyprotein.

### 2.2.2 CBSV transmission

Lister (1959) reported successful sap transmission of CBSV to herbaceous host plants from cassava infected leaves and isolates from infected host plants were back transmitted to susceptible cassava after several trials. Bock *et al*. (1978) confirmed the Lister (1959) findings on sap transmission of CBSV. In the field CBSV is perpetuated in three ways; by planting infected cuttings, by whiteflies transmission and graft-transmission (Storey, 1936). Storey (1939) earlier stated that CBSD was caused by a virus whose vector was likely to be whitefly because by then *B. tabaci* was already established as the vector of cassava geminiviruses. But an attempt to prove this by Bock (1994) was not successful. Transmission using the aphid *Myzus persicae*, five other species of aphids and mealybugs also failed (Storey, 1947; Lennon *et al*., 1986), which created some uncertainty over the vector. Despite the failure, speculation on whitefly involvement continued. Cassava clones introduced from other areas of Africa or South America known to be free from CBSD, however, became infected in CBSD endemic areas which indicated to Hillocks and Jennings (2003) that the virus is indeed transmitted naturally. Moreover, cassava plants obtained from Africa and raised from seeds at different sites in Mozambique, Kenya and Tanzania also became infected (Calvert and Thresh, 2002).

Field observations in Tanzania during the 1990s indicated a close association between the incidences and spread of CBSD and whitefly population changes (Hillocks *et al.,* 1999). The first recorded successful vector transmission of CBSV was achieved in a glasshouse and insectaries at NRI between CBSV-infected cassava and virus-free cassava plants (Maruthi *et al*., 2005), although at low rate

(22%). Maruthi *et al* (2005) further pointed out that the feeding behaviour of *B. tabaci* on cassava plant may influence CBSV transmission and that *B. afer* and the spiralling whitefly (*Aleurodicus dispersus*) Russel might also transmit CBSV under suitable conditions. This was later confirmed by Mware *et al*. (2009).

### 2.2.3 CBSD symptoms

The first description of CBSD symptoms was by Storey (1936). CBSD symptoms are unusual in that they affect all parts of the plant; stems, leaves storage roots and fruits (Hillocks *et al*., 1999). On the stem during periods of dry cool weather, the disease can cause shoot die back and necrotic lesions. CBSD symptoms are expressed as brown lesions, which appear on the young green stem, and these were first regarded as the most conspicuous symptom of the disease. However, Hillocks *et al.* (1996) noted that this symptom is not the only prominent symptom and it is often absent. Nichols (1950) distinguished foliar chlorosis symptom associated with CBSD at Amani in northern Tanzania and presented a more comprehensive description of the disease. Plants may be infected with CBSD but disease incidence and severity depends on the environmental condition, growth stage of the plant, time of infection and varietal sensitivity (Hillocks, 1997). CBSD symptoms can be masked by CMD symptoms particularly where both diseases and green mite attack plants. Both CMD and CBSD show foliar chlorotic symptom but unlike CMD, in which symptom expression occurs on young leaves, CBSD symptoms show varying patterns of chlorosis on the old leaves (Figure 2.1a) and do not cause distortion on the lamina (Hillocks, 1997).

**Leaf symptoms**: In his work, Nichols (1950) described certain types of foliar chlorosis associated with CBSD and these were further explained by Hillocks (1997); (1) a leaf chlorosis which starts along the margins of secondary veins expanding to the tertiary veins and finally produces chlorotic blotches. (2) A chlorosis which develops in roughly circular patches between the main veins and may affect much of the lamina. This type is the most common in which smaller leaves of the severely affected plants present a striking appearance in contrast to the fully green young leaves (Hillocks and Thresh, 1998). Disease symptoms are not present on newly formed foliage during hot seasons (Hillocks, 1997).

**Stem symptoms**: Stem symptoms may not always be associated with CBSD, except in highly susceptible varieties (Hillocks, 1997). Purple/brown lesions may be observed on the outer-surface which is seen to have penetrated into the cortex. When the outer bark is stripped, the necrotic lesions in the leaf scars became prominent after leaves shedding due to normal senescence. In severe infections, these lesions kill the dormant auxiliary buds. This is followed by general shrinkage of node and death of internode tissue leading to branch necrosis from the tip downwards, to cause what is known as 'die back' (Figure 2.1b).

**Root symptoms**: Symptoms vary on the outside of the storage root and may occur as radial constrictions in the surface bark. Tissue surrounding this constriction is stained brown or black under which the cortex is necrotic. The internal symptoms consist of yellow/brown, corky necrosis of the roots or with black streaks (Hillocks *et al*., 1996). In sensitive varieties, almost the whole of the roots may be affected (Figure 2.1c). In advanced stages, the presence of secondary organisms, decay and soft rot may occur. Symptoms on roots usually develop after leaf symptoms and the latent period of root necrosis is variety specific. Root symptoms occurred eight months after planting (MAP) in certain varieties, despite the earlier presence of symptoms on leaves (Hillocks *et al*., 1996). In susceptible varieties, where CBSD-infected cuttings were used as planting materials, root symptoms were observed 5-7 MAP (Hillocks, 2003).

In a survey conducted by Legg *et al.* (1994) only the leaf and stem (Figure 2.1d) symptoms but not the root symptoms were seen (Jennings 1960b; Thresh *et al*., 1994). Hillocks (1997) noted that there may be recovery from leaf and shoot symptoms during the active period of plant growth. Studies in Tanzania showed that greater than 90% of the susceptible varieties obtained from diseased stems showed leaf symptom at the time of sprouting, while many of the same plants 12-59% (depending on the varieties) expressed root symptoms during harvest (Hillocks *et al*., 2001).

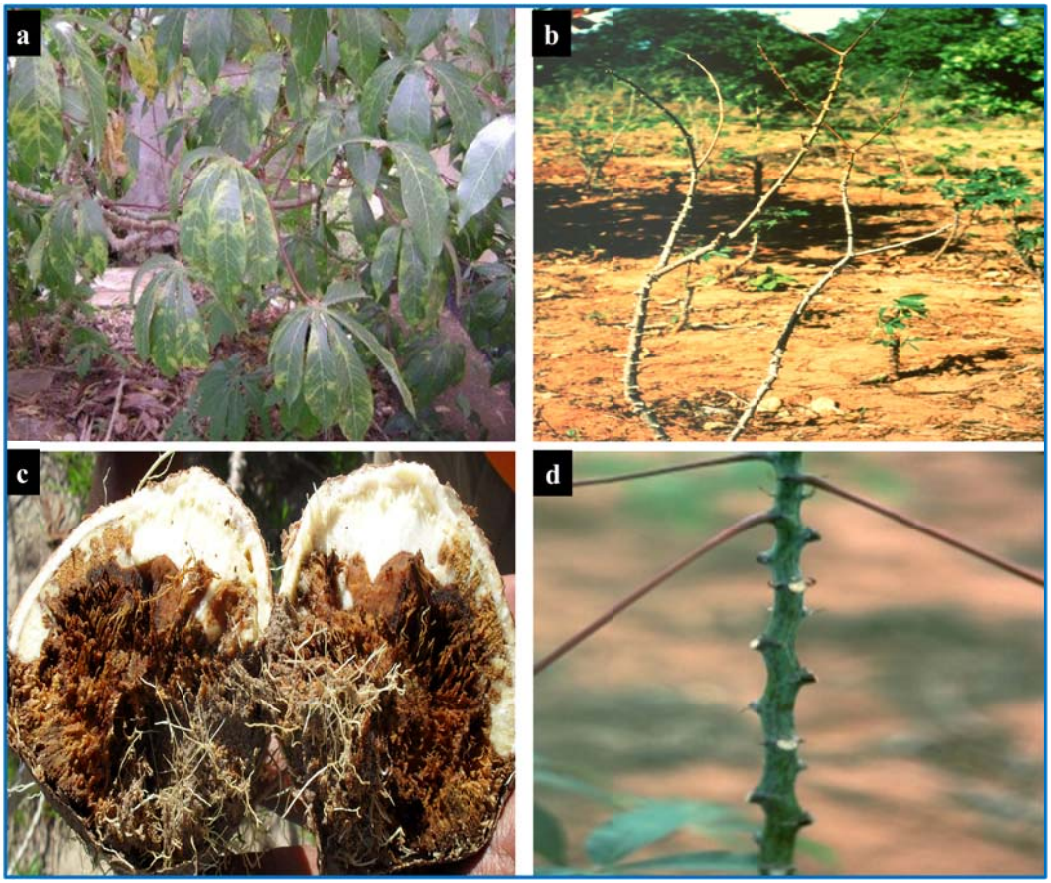



**Figure 2.2:** CBSD symptoms in different parts of cassava plant (a) leaf symptoms, (b) plant dieback, (c) storage root symptoms and (d) brown streak lesion on the stem. Photos adopted from (R.J. Hillocks).

### 2.2.4 The whitefly vector, *Bemisia tabaci*

The whitefly, *B. tabaci*, is an insect pest whose larvae and adults obtain nutrient by sucking juices from plant leaves and stems. There are approximately 1500 species of whiteflies (Martin *et al.,* 2000), which are under two sub-families *Aleuordicinae* (mostly found in South America) and *Aleyrodinae* (more widespread) (Ko, 2001) of the family *Aleyrodidae*. Adult whiteflies measure from 1-3 mm in length, have four wings are seven segments, fully mobile with a feeding rostrum and antennae (Martin *et al.,* 2000). Biotic variations of whiteflies including host preference, host range, fecundity and dispersal behaviour of the vector may likely influence vector-host interactions in cassava (Burban *et al*., 1992; Brown *et al*., 1995; Maruthi *et al*., 2005). Fecundity of *B. tabaci* can be estimated by counting the number of eggs oviposited by the female every 48 hour (Maruthi *et al*., 2005); while survival or longevity is the maximum time in days that an adult female *B. tabaci* lived on a plant (Perring, 2001). Whiteflies damage

crop plants in four ways: mechanical damage through feeding, secretion of honeydew, physiological disorders (Martin *et al.*, 2000) and transmission of viruses (Maruthi *et al*., 2005; Mware *et al.,* 2009). In addition, virus diseases associated with whiteflies were also reported in non-crop plants in the tropical and non-tropical agro-ecosystems (Verma, 1963; Bock, 1982).

Most plant viruses depend on vectors for plant-to-plant spread (Ng and Falk, 2006). Over 80% of plant viruses depend on insects for transmission (Holn, 2007). The piercing-sucking mouth parts of insects such as aphids, whitefly and leafhoppers facilitate efficient extraction of plant sap as well as transmission of plant viruses. Crop plants typically infected by whitefly-transmitted viruses in Eastern Hemisphere are cassava, brassicas, tobacco, tomato, legumes (*Vigna* and *Phaseolus)* species (Muniyappa, 1980; Mound, 1983); in the Western Hemisphere are bean (*Phaseolus vulgaris*), cotton, soybean and tobacco (Bird, 1978; Brown, 1990; Polston, 1997; Paximadis *et al.,* 1999).

About 144 plant viruses are transmitted by whiteflies, of which *B. tabaci* transmits 111 and 33 by two other species of whiteflies; *Trialeurodes vaporariorum* and *T. abutilonia* (Jones, 2004). *B. tabaci* is polyphagous feeding on over 500 species of plants (Brown *et al.,* 1995) and thus has the potential to transmit viruses to a wide range of host-plants. About 90% of the whitefly transmitted virus species belong to the genus *Begomovirus*, 6% *Crinivirus* and 4% belong to the remaining genera in the *Closterovirus* and *Ipomovirus* (Jones, 2004).

### 2.2.5 Virus-vector interactions

CMVs and CBSVs are the most damaging whitefly transmitted viruses of cassava in Africa (Bock, 1982; Legg *et al*., 1994; Maruthi *et al.,* 2005; Mware *et al.,* 2009). Earlier studies have shown that *B. tabaci* vector variants differ in ability to transmit certain viruses and transmission can be more or less effective (Bird, 1957). It was earlier believed that *B. tabaci* does not adapt well to elevation above 1000 m a.s.l. (Morales and Aderson, 2001), and thus believed to be outside the zone of CBSD. However, plenty of whiteflies were observed recently in the CBSD epidemic areas of Uganda and Lake Victoria at altitudes at least up to

1300 m a.s.l and 1100 m a.s.l., respectively (Legg *et al*., 2011). In addition, *B. tabaci* is widely adapted in a region extending from more than 30 $^{o}$S and 40 $^{o}$N and this limit does not relate to temperature which seems to vary widely over the altitude range (Mware *et al*., 2009).

The interactions between virus and vector during transmission are very specific (Ng and Falk, 2006). This interaction is believed to be mediated through capsid and helper components of certain viruses (Pirone and Thornbury, 1988; 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 acquisition access period (AAP) and the inoculation access period (IAP) required for the interactions have led to three different categories of vector-transmitted viruses (Ng and Falk, 2006).

a) Non persistent, stylet-borne (occurs within few minutes to hours of feeding), b) semi-persistent, foregut-borne (hours to days) and c) persistent, circulative (days to months or even years). In addition a 'propagative' form of virus transmission, in which the virus passes to the vector's progeny, was also described as the fourth category (Nault, 1997).

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 such as UCBSV and 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 (semi-persistent). 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. The interaction between CBSV and *B. tabaci* is semi-persistent. Maruthi *et al.,* (2005) reported a 48 h each for AAP and IAP for successful CBSV-transmission. The semi-persistent interaction between *B. tabaci* and a 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 *B. tabaci* are lacking. However, the involvement of the capsid protein (CP) as reported in vector-based transmission of CVYV (Janssen *et al*., 2005) could be characteristics of CBSV.

### 2.2.6 Economic losses due to CBSD

CBSD causes up to 70% losses in root weight in some sensitive varieties (Hillocks *et al*., 2001). The poor quality of the tuberous roots resulted in a greater loss of economic yield (Nichols, 1950). The extent of weight loss was dependent on the earliness of maturity of the tubers and the relative susceptibility of the varieties to the virus (Hillocks and Jennings, 2003). The success in overcoming the CMD pandemic that ravaged Uganda in the 1990s is somewhat overshadowed because many varieties resistant to CMD are now found susceptible to CBSD. Alicai *et al.* (2007) reported that in Mukono district of Uganda, one out of four farmers' field planted with cassava variety 92/0057, which is known to be resistant to CMD showed symptoms of CBSD. The impact of CBSD is said to affect 20 million people in rural communities in the areas where the disease is endemic (Legg and Hillocks, 2003; Hillocks, 2005). Hunger and the lost household income have left many families in total dilemma (Pearce, 2007). CBSD has turned the long-term chronic food shortage in Malawi and Mozambique into an acute one (Shaba *et al*., 2003; Steel, 2003). In Malawi, farmers adopted early harvesting and selective harvesting to minimize the impact of CBSD on yield loss (Hillocks *et al*., 2001; Gondwe *et al.,* 2003), implying a great challenge to the quality of cassava as a food reserve. The likely negative impact of these harvesting methods is that the diseased plants left in the field become the pool for next season's planting material. Stem necrosis decreases the viability of cuttings, leading to low plant populations.

In southern Tanzania, CBSD is reported to render 20 to 80% of roots unusable for human consumption (Katinila *et al*., 2003). Gondwe *et al.* (2003) and Shaba *et al.* (2003) also reported a yield loss of 18 to 60% in Malawi. CBSD caused huge economic losses in these areas. For example, the annual yield loss caused by CBSD in Malawi was estimated to be over 1.4 million tonnes of cassava, which translates to US $7 million (Gondwe *et al*., 2003). For CMD, loss assessment has been fully documented and total cassava losses due to CMD in Uganda were estimated to be about 24% (Gibson *et al.,* 1996; Pita *et al.,* 2001; Legg and Thresh, 2003). Under favourable conditions, CBSD was said to cause total loss of the cassava crop which CMD rarely does (Legg *et al.,* 2011). The first recognized effect of CBSD was on the development of cuttings, because the disease destroys

many of the buds and infected cuttings often fail to produce shoots (Storey, 1938). In experimental plots at Mvuazi, DRC, high incidences of leaf symptoms and root necrosis of up to 100% were recorded (Mahungu *et al*., 2003).

Areas ravaged by CBSD in Mozambique have experienced food insecurity (McSween *et al*., 2006). Up to 100% yield loss was recorded in Mozambique due to the impact of CBSD (Hillocks, 2005). This posed a serious threat to the livelihood of people living in this area. For instance Mogincual District in Mozambique, where a variety called Calamidade was grown and farmers obtained nothing but a mass of rotting tuber tissue due to root necrosis caused by CBSD (McSween *et al*., 2006). The threats posed by CBSD forced poor farmers to harvest cassava before reaching full yield potential and discourage the storage ability of the crop in the field for long (Hillocks and Jennings, 2003). Yield losses of up to 60% from CBSD root necrosis were estimated in Masasi District of Mtwara Region in Tanzania (Kanju, 1989).

A moderate infection by CBSD (10-30% damage to root surface area) decreases the market value drastically by 90%, fetching under $5 per tonne, as opposed to US $55 for fresh healthy cassava root. A severe disease completely destroys roots and makes them unfit for market or even own consumption by farm family (McSween *et al*., 2006). Current estimates indicate that CBSD causes economic losses of up to $100 million annually to African farmers (IITA, 2005). Root necrosis, constriction and pitting cause primary yield losses, while secondary losses arise from the reduced number of roots due to CBSD (Gondwe *et al*., 2003; Hillocks *et al*., 2001; Kanju *et al*., 2003a).

### 2.2.7 Control methods

Attempts to understand and provide adequate control measures to virus diseases of cassava, through selection and use of resistant cassava cultivars date back to 1927 (Storey, 1938). Hillocks (2002) in Tanzania advocated screening of local landraces as a rapid way of obtaining locally adapted varieties with resistance to CBSD. Sanitation techniques could be used which include taking cuttings from healthy plants only and subsequently removing any plant which is diseased (roguing), as well as cultural practices (Kanju *et al.,* 2003a). Cultural practices

such as good farm hygiene and removal of weeds around cassava farm could be recommended in the control and management of whitefly since many weed species are hosts to whitefly. However, sanitation has its own limitations in that the disease free material selected is no more resistant than its parent stock, re-infection may also occur in areas of greater disease pressure and the excessive rouging will result in a limited crop stand (Bock, 1983 and Fargette *et al.,* 1985).

The effectiveness of sanitation depends on the inoculum pressure. Hillocks (2002) suggested disease incidence of below 20% for roguing to be effective. It was also believed that sanitation has an important role in the development of an integrated strategy for the control of CBSD (Legg and Thresh, 2003). In Uganda, roguing has been used to eradicate CBSD in the past (Jameson, 1964). In addition, Mtunda *et al.* (1998) reported the use of roguing in Tanzania to produce breeding stocks from cassava plants initially showing symptoms of CBSD. However, these measures are not fully implemented for various reasons itemised by Hillocks (2003): 1) Farmers have difficulty in recognising CBSD symptoms due to variability in symptom expression. 2) Planting material is taken at different times of the year and often it is in scanty supply, limiting the ability to select disease-free material. 3) Farmers are reluctant to rogue since roguing lowers plant density, thereby resulting in less yield (Kanju *et al.,* 2003b).

In East Africa, selection for increased resistance was seen as an option (Storey, 1936) although resistance is lacking for CBSD. Emphasis was given to the need to transfer resistance to cassava from related species such as *Manihot glaziovii* Muell-Arg., *M. catingae* Ule, and *M. dichotoma* Ule (Hillocks, 2003). The recent trend in the selection for resistance employed farmer participatory selection as a key to development of new varieties that are resistant to CBSD (Kanju *et al.,* 2003a). The following varieties tolerant to CBSD were identified in Tanzania: Nachinyaya, Kiroba, Kigoma Red, Namikonga, Kitumbua, Kalulu, Kikumbe, UKG 93/041, TMS 8475, TMS 82/0061 and Naliendele 34 in Mozambique, Nikwaha, Mulaleia, Chigoma Mafia, Mwento, Waloya, Binte Massuea, MZ89001 and MZ89186, in Malawi, Gomani, Kirobeka, Nyankwazi, CH95/196, CH95/102, BA95/070 and MK96/054 (Hillocks, 2006). Resistance in some of these earlier

selections was reported to be broken down subsequently (R.J. Hillocks, personal communication, 2010).

Recent studies have associated the increase in whitefly populations with greater incidences of CBSD in Uganda (Alicai *et al.,* 2007), Tanzania (Robertson, 1987; Hillocks and Jennings, 2003; Maruthi *et al.,* 2005) and Malawi (Legg and Raya, 1997). Severe CMD pandemic that spread from Uganda to neighbouring countries since the 1988 was also linked to greater population of *B. tabaci* (Deng *et al.,* 1997; Legg, 1994; Otim-Nape *et al*., 2001). There were concerns that the large whitefly numbers that have persisted on selected cassava varieties in Tanzania such as Naliendele 34 may affect the stability of resistance to CBSD. Little attention is given to control CMD or CBSD by managing whitefly vector as has been the case for other virus diseases, such as cotton leaf curl virus and tomato yellow leaf curl virus which targeted both the viruses and the vector *B. tabaci* (Rapisarda and TropeaGarzia, 2002).

Chemical pesticides, biopesticides such as Bt and NPV, use of natural enemies and physical barriers have been used to control *B. tabaci* on cassava in Latin America (Belloti, 2002), but in SSA this has not been attempted for economic reasons. It's an expensive strategy to many resource poor farmers in Africa and also insecticides are not readily available (Hillocks, 2002). Moreover, chemical control is only effective where the vectors feed on a crop for several hours before the virus is transmitted. If the virus transmission occurs with minimal feeding time, infection is likely to occur before the vector is killed by the insecticide (Ahmad *et al*., 2003). A number of insecticides have effectively controlled pests in the past but many pests have now developed resistance.

Parasitoids could be used in biological control of whitefly. Biological control of the vectors can be very effective but the cost of producing and releasing natural enemies is very high (Hillocks, 1997). Seed propagation may control viral diseases as seeds of some viral infected plants may be virus-free but this may not be an option in some crops like cassava because of the high variability in the seed derived progeny (Ekanayake *et al*., 1997). The need to put in place strict control measures was advocated in order to check the movement of cassava germplasm

from one geographical location to another (Kanju *et al.,* 2003b). Legg and Thresh (2003) reported that Africa's major cassava producers such as Nigeria, Ghana, Benin and Cote d'Ivoire seem to have favourable environments for CBSD. Therefore control of the movement of cassava cuttings from one country to another should be strictly regulated.

**Use of resistant varieties**: Virus diseases cannot be chemically controlled the way some fungal or bacterial diseases are (Hillocks, 2002). Therefore, strategies for viral disease control focus on preventive measures, provided such measures are simple, inexpensive and within the limited capacity of the farmers. This can be achieved in diverse ways which include quarantine measures, early harvesting, use of resistant varieties and use of virus-free planting material.

Strict quarantine measures are effective in disease free areas (Legg and Thresh, 2003). Early harvesting of cassava was practiced by farmers in Mozambique and Tanzania to avoid CBSD from destroying the roots (Hillocks *et al*., 2002). However, this strategy threatens the role of cassava as a famine reserve crop as it cannot be left in the field as a food reserve (Kanju *et al*., 2003). The use of resistant varieties is recommended for managing CBSD (Storey, 1939; Hillocks and Jennings, 2003), especially where the disease pressure is high (Hillocks and Thresh, 2003). For example, Nanchinyaya, Namikonga and Kiroba in Tanzania, which were the local tolerant varieties identified and recommended to farmers (Hillocks *et al.,* 2001; 2003; 2005; 2006; Kanju *et al*., 2003a).

Resistant varieties have obvious advantages in decreasing the losses due to viral diseases (Nichols, 1947). Resistant cultivars can be developed through conventional breeding programmes or through transformation with resistance genes (Okogbenin *et al*., 2007; Takeshima, 2010). Resistance genes for CBSD can be transferred from cassava related species, such as, *M. glaziovii, M. dichotoma*, *M. catingae* and 'tree' cassava, believed to be a natural hybrid between *M. esculenta* and *M. glaziovii* (Nichols, 1947; Jennings, 1957; Allem, 2002). A few cassava cultivars such as Macaxiera Alpin are resistant to CBSD (Jennings, 1957). Another two shrub-like species *M. saxicola* and *M. melanobasis*

are also highly resistant to CBSD but their roots contain high concentration of hydrocyanic acid (Jennings, 1957).

A limitation with conventional breeding for resistance to CBSD is that it is laborious and requires much time. Each generation takes not less than three years and a series of backcrosses are needed to remove the undesirable characteristics such as tree like characteristics and high cyanide level while retaining resistance to CBSD (Jennings, 1957). Another limitation is that crops are usually infected by several distinct viruses (Mukasa *et al.,* 2003) and this might require several separate gene incorporations.

**Reversion in cassava varieties:** Reversion was first reported for virus infection in the 1930s when symptomatic cassava varieties infected with ACMV sprouted without CMD symptoms (Storey and Nichols, 1938). Since then a number of researchers have observed and confirmed reversion (Gibson and Otim-Nape, 1997; Fondong *et al.,* 2000). In addition varietal differences have also been reported to influence reversion in cassava plants (Jennings, 1960b; Rossel *et al*., 1992; Thresh *et al*., 1994; Fargette *et al*., 1996; Gibson and Otim-Nape, 1997).

**Use of virus-free planting material**: Viruses can be transferred between generations in crops which have seed-borne virus diseases or which are vegetatively propagated, such as cassava (Mtunda *et al*., 1998). In the absence of resistant cultivars, the benefits of selecting virus-free stems when replanting cannot be overlooked towards the control of viral diseases. Currently there are no cultivars resistant to CBSD and many cultivars such as TME 14, TME 204, NASE 10, NASE 12, I95/0087 and I92/0057 have been bred and selected for yield, quality and resistance to CMD but are highly susceptible to CBSD (Alicai *et al*., 2007). In such a situation, use of virus-free planting material remains a hopeful alternative.

The major drawbacks with selecting virus-free material are; (1) possibility of re-infection and the difficulty that farmers, extension workers or even researchers can face in correctly identifying virus-free planting material by visually looking at the symptoms (Hillocks, 1997). CBSD foliar symptoms are often not clear and

normally occur only on mature leaves. The young expanding leaves commonly appear symptomless (Hillocks *et al.,* 1999). Several diagnostic and virus elimination techniques are now available for testing and free planting material from viruses/diseases. Virus-free planting materials are of little value in areas of high CBSD incidence, because provided whitefly numbers are sufficient, re-infection from the surrounding cassava will be rapid.

### 2.2.8 Plant infectivity assays

Different plants vary in their susceptibility to viruses and in their ability to show clear and distinctive symptoms after infection with different viruses. Those which show clear symptoms are known as indicator plants (Lister, 1959). The choice of indicator plants depends on the virus and species, those commonly used include *Chenopodium quinoa, C. amaranticolor, Gomphrena globosa, Ipomoea setosa, Phaseolus vulgaris* and *Nicotiana species*. For CBSV, *Petunia hybrida, Nicotiana debneyi, N. benthamiana, N. tabacum* and *N glutinosa* (Lister, 1959). The optimal stage of growth at which the indicator plant is used also varies depending on indicator plant species. Most *Nicotiana* species are used at the four leaf stage. The leaves of beans are very sensitive to some viruses whilst plants like *Chenopodium* can be used up to the ten leaf stage (Bock, 1994).

Several methods for inoculating plants are available which include grafting, use of dodder plants, use of vector and sap inoculation (Boissot *et al.,* 2008). Since viruses systemically infect their hosts, they can be inoculated through graft unions between diseased and healthy plants by allowing vascular union between stock and scion (Idris *et al*., 2001). Graft inoculation has been used for inoculating Yellow vein mosaic virus in okra (*Abelmoschus esculentus*) (Boissot *et al.,* 2008) and ACMV and CBSV in cassava (Ogbe *et al*., 2002). Although graft-inoculation may be limited to plants that are closely related, plants like solanaceous species of tobacco, tomato, potato and thorn apple are also graft compatible (Akhtar *et al*., 2003). With sap inoculation, virus suspension in sap from infected plants is introduced into healthy indicator plants. Purified virus preparation is preferred although inoculation can usually be achieved with crude sap (Mumford *et al*., 2006; Boissot *et al.,* 2008; Ogwok *et al.,* 2010). Inoculum can be applied in various ways, for example by stroking the plants with a virus contaminated

finger, piece of muslin, soft brush or by a spray gun which injects inoculum deeply into the tissues of the host plant.

The rubbing method involves using an abrasive such as carborundum or celite to produce entry wounds on the leaves of indicator plants (Mumford *et al*., 2006). The abrasive is either added to the homogenized tissue of infected plants or can be blown onto leaves of indicator plants before inoculation (Lister, 1959). Susceptible indicator plants may react by forming localized lesions on the inoculated leaves which normally appear in 4-7 days or by showing systemic symptoms on the youngest leaves in a week or more (Lister, 1959). The period before symptoms appear on indicator plants can be influenced by the amount of inoculum applied and the temperature (Lister, 1959; Ogwok *et al.,* 2010). It is therefore important that the plants be kept long enough to allow the symptoms to appear. The limitation with plant infectivity technique is that it requires more time to perform than the serological assays.

### 2.2.9 Electron microscopy

Because of their small size, all virus particles can only be visualised using an electron microscope (MacRae and Mukesh, 1998). Elongated virus particles such as CBSV which are flexuous rods and other rod shaped or filamentous were more readily distinguished than spherical ones (Kitajima and Costa, 1964; MacRae and Mukesh, 1998). However, this technique is only reliable if relatively high concentrations of viruses are present (MacRae and Mukesh, 1998).

### 2.2.10 Enzyme linked immunosorbent assay

Enzyme immonosorbent assay (ELISA) is very sensitive as it can be used to detect as little as 11 ng of virus. ELISA is suited to testing large number of samples and can be use for quantifying the virus as well (Hammond *et al*., 1992). Different types of ELISA are available depending on the number of antibodies used during the reaction (Abouzid *et al*., 2002). By using different antibodies, it is possible to test plants for different viruses (James *et al*., 2006). However, antibodies that can detect some viruses such as CMV and CBSV are readily available but antibodies for the detection of some viruses like SPLCV are not yet

developed. ELISA is less sensitive than PCR especially if the virus titre in the sample is low (Hu, 1995).

### 2.2.11 PCR-based detection of viruses

PCR detection techniques are highly sensitive and for CBSV, RT-PCR technique can detect the virus in young leaves of cassava that are not yet showing symptoms (Abarshi *et al*., 2010). PCR techniques, however, require that the sequence of the viral genome is known and two small sections of 20 or less nucleotide bases are chosen and used to produce the primers (Gibbs and Mackenzie, 1997). Some primers can be designed using regions of the viral genome which are conserved among viruses of one group and these can be used to detect more than one virus within the group (Chen and Adams, 2001). The use of reverse-transcription quantitative PCR (RT-qPCR) has revolutionized PCR based detection of viruses. The technique is more efficient (90% detection by RT-qPCR *versus* 45% detection by conventional RT-PCR) (Kokkinos and Clark 2006). Despite this, RT-qPCR equipment is not commonly available in Africa and requires expensive consumables.

**Use of RT-PCR for the detection of UCBSV and CBSV:** The molecular technique for CBSV and UCBSV detection using RT-PCR was first developed by Monger *et al.,* (2001a). Using CBSV gene-specific primers, the virus was isolated from infected cassava samples and sequenced. Using this technique, possible occurrence of two CBSV strains was described (Monger *et al.,* 2001b). The robustness and high sensitivity of the RT-PCR technique has promoted its wide use. In East Africa, the technique was used effectively to detect and confirm the presence of CBSV in infected plants (Alicai *et al.*, 2007). Since CBSD symptoms are often unclear RT-PCR based detection can be used to ensure that starting material for cutting multiplication schemes is virus-free. To achieve this, systematic virus-testings are recommended especially for experimental purposes and primary multiplication sites (Abarshi *et al*., 2010). Detection of both CBSV and UCBSV in a single RT-PCR was first described by Abarshi *et al*. (2010). In Uganda, a new technique was developed and used for the detection and discrimination of the two viruses by a single RT-PCR test and this can be used to

study for mixed infections of UCBSV and CBSV in East Africa (Mbanzibwa *et al*., 2011).

### 2.2.12 Virus elimination techniques

Several methods are employed to eliminate viruses from propagation material. These include electrotherapy, chemotherapy, thermotherapy, cryopreservation and tissue culture methods. However, virus elimination is an extremely pathogen/host dependant process and no generalizations can be made (Lizarraga *et al*., 1980). *In vitro* micro propagation of cassava has been achieved in several studies.

In a study conducted by Korean (2003) it was observed that adventitious roots and shoots from the explants of cassava differentiated more efficiently in liquid medium than in solid medium (Kaiser and Teemba, 1989). Root formation was inhibited by callus forming on the cut-end of the node cuttings on medium with zeatin at high concentrations (Ezeibekwe *et al.*, 2009). On the other hand, root formation was not inhibited in a medium supplemented with NAA and kinetin at low concentration (Encina *et al*., 2001). *In vitro* thermotherapy has been successfully used for virus elimination in several crops. Leonhard *et al.* (1998) reported successful eradication of virus from Australian grape vine varieties. Thermotherapy has also been a successful method for eliminating several viruses in sweet potato, potato and cassava (Kaiser and Teemba, 1989; Griffiths *et al.,* 1990; Meybodi *et al*., 2011). Heat therapy of cassava for eliminating CMBs was achieved *in vitro* at 37$^{o}$C for 6 weeks under a regime of 16 h light and 8 h dark (Kaiser and Teemba, 1989). Walkey (1976) also observed complete eradication of *Cucumber mosaic virus* (CMV) from *Nicotiana rustica* when cultures were kept continuously at 32$^{o}$C for 25 days under a 16 h light and 8 h dark period. Inactivation of CMV in cultured *N. rustica* by alternating diurnal periods, 40 $^{o}$C for 16 h of light and 22 $^{o}$C for 8 h of darkness for 12 days was later proposed by Walkey and Freeman (1977).

Thermotherapy for the elimination of CBSV from cassava was carried out directly on fully grown *in vitro* cassava plantlets (Wasswa *et al.,* 2010). The success of elimination of viruses depends on the type of plant viruses, the hosts

(varieties) and if the plant is single or mixed infected (Zapata *et al.,* 1995). Temperature and time of exposure may be complicated by heat tolerance of the cassava varieties. It is therefore, important to select a temperature regime which is above the optimum for growth, but not lethal to the plant.

Another method used for virus eradication is chemotherapy. The incorporation of ribavirin (1-ß-ribofuranosyl-1, 2, 4-triazole-3-carboxamide), which is an anti-metabolite chemical; in the tissue culture medium has been studied (Cassells and Long, 1982; Klein and Livingston, 1982; Nascimento *et al* 2003; Mahfouze *et al.,* 2010). Ribavirin has been shown to have some activity against virus replication in humans (Sarver and Stollar, 1978) and plants (Walkey, 1985). Some virus-free plants from CMV- and PVY-infected tobacco explants were regenerated using ribavirin (Cassells and Long, 1982). The simultaneous application of chemotherapy and thermotherapy methods has been also efficient for eliminating viruses in potatoes (Nascimento *et al* 2003). However, anti-viral chemicals (such as ribavirin) can be toxic which can inhibit host development (Sarver and Stollar, 1978; Elia *et al*., 2008).

### 2.2.13 Mechanisms of resistance to virus infection

Mechanisms of resistance to CBSV are not fully understood, although Nichols, (1950) and Jennings (1960b) speculated that resistant cassava varieties are likely to localise the virus in their roots. Wilson and Jones (1992) reported that the mechanism of resistance to plant viruses involves resistance to the phloem transport of viruses. However, resistance to viruses may involve one or all of the following itemised mechanisms, described by Solomon-Blackburn and Baker (2001) as follows:

1- Plants rapid defence cause by hypersensitive reaction (HR) that resulted in the necrosis of few cells at the site of infection, preventing spread of infection to other areas.
2- Prevention of virus multiplication at the early stages of infection called extreme resistance (ER), but this is not normally associated with the death of cells.
3- Plants being unattractive to vectors or resist virus infection.

4- Resistance to virus accumulation, where plants are infected, but the virus accumulation is very low in the plant and restriction of virus movement from inoculation sites to other parts of the plant.

**RNA silencing**: Mechanism of resistance employed by plants against the foreign genes entering the plant is referred to as gene silencing (Waterhouse *et al*., 2001; Vaucheret, 2001; Voinnet, 2001). Foreign RNAs are degraded by the endoribonuclease Dicer into small effector molecules called siRNAs (small interfering RNAs) (Waterhouse *et al*., 2001). Dicer was originally identified as a nuclease involved in the RNA interference (RNAi) pathway of animals (Bernstein *et al*., 2001). The method of siRNA is triggered by long double-stranded RNA (dsRNA) (Fire *et al*., 1998). The dsRNA trigger is cleaved by Dicer into 22-nt RNAs (Elbashir *et al*., 2001). The 22-nt RNAs, known as small interfering RNAs (siRNAs), act as guide RNAs to target homologous mRNA sequences for foreign RNAs degradation (Bernstein *et al*., 2001). Typically siRNAs are incorporated into RNA-induced silencing complex (RISC) which consists of several proteins including the Argonaute (AGO) protein (Elbashir *et al*., 2001). RISC is presumably located in the RNA degradation center in the cytoplasm (Bernstein *et al*., 2001). After RISC-mediated mRNA cleavage, the resulting degraded products are further subjected to the exonucleolytic degradation (Ratcliff *et al*., 1997). Threfore, plants combat virus infection by gene silencing, a general mechanism normally used for maintaining homeostasis (Covey *et al*., 1997; Ratcliff *et al*., 1997). On the other hand, viruses attempt to suppress host gene silencing at an early stage of infection (Brigneti *et al*., 1998; Jones *et al*., 1998; Kasschau and Carrington, 1998). Gene silencing is either at the post-transcriptional level, in which the silencing mechanism targets mRNA before it is translated into respective proteins (Hamilton and Baulcombe, 1999; Dalmay *et al*., 2001) or transcriptional level (Vaucheret, 2001), in which RNA silencing is before transcription. Here the gene is made unattainable to transcriptional machinery by RNA silencing mechanism (Baulcombe, 1996). The silencing system is very specific and precise, degrading only foreign and unusual mRNA, at sites of infection, followed by a systemic signal sent to distal parts of the plant to degrade any particles homologous to mRNA perceived by the plant to be abnormal (Ruiz *et al*, 1998; Llave *et al*., 2002). Another pathway which seemed

to be similar to RNA silencing s involves the use of microRNAs (miRNA) (Carrington and Ambros, 2003). Using these pathways as basis, development of transgene-based control techniques for CBSV and UCBSV and testing of target strategies has been initiated by Patil *et al*. (2010). It was advocated that such studies should focus on incorporating transgenes conferring robust CBSD-resistance into conventionally bred CMD-resistant varieties (Legg *et al*., 2011).

### 2.2.14 Evaluation of CBSD resistance

Breeding for resistance to cassava viruses is posed with the problem of researchers not having standard terminologies in evaluating for resistance. Lapidot and Friedmann (2002) reported that while, breeders emphasise the effect of resistance on yield and quality; pathologists consider the fate of the virus in the plant. In the past many attempts have been made to evaluate resistance to CBSD (Nichols, 1947; Jennings, 1957; 1960b; 2003). Hillocks *et al.* (1996) described a scoring scale of 1 to 5 to score for CBSD symptoms severity of leaf and stem. In addition, Hillocks and Jennings (2003) described two other approaches for evaluating resistance to CBSD. The first approach involves planting cuttings from CBSD symptom-free plants and growing them in hot spot areas to permit substantial plant-to-plant transmission of viruses. A new incidence of leaf and stem symptoms are recorded monthly and root necrosis is recorded at harvest. The second approach involves evaluating cassava varieties for resistance to infection with CBSV based on four resistance groups as follows:

1- Resistant cassava varieties that remained symptom-free after exposure to infection

2- Moderately resistant, in which varieties developed mild symptoms in a few plants

3- Slightly resistant, in which varieties developed CBSD symptoms in over 90% of the plants. However, the symptoms are mild, or restricted to the stem or leaves in 40% of plants

4- Susceptible, in which cassava varieties expressed severe CBSD symptoms in all the plants (99% of the plants).

5- Reversion, in which virus free plants are obtained from plants of CBSD-infected cuttings grown.

## CHAPTER 3: General materials and methods

General materials and methods common to this study are explained here while specific details for each study are given in respective Chapters (4-7).

### 3.1. Cassava varieties and growth conditions

Plants used in this study were obtained as stem cuttings of six disease-free cassava variety of Kaleso (from Kenya), Ebwanatereka (from Uganda), Albert and Kiroba (both from Tanzania) from farmer's fields. Cassava variety Columbian was obtained from the University of Bristol, UK, and TMS60444 from the International Laboratory for Tropical Agricultural Biotechnology (ILTAB), St. Louis, USA. Plants were grown at 28 ± 2 $^{o}$C, 50-60% relative humidity (RH) in the quarantine glasshouse and observed for CMD and CBSD symptom expression. Plants were tested using RT-PCR tests and the absence of CBSV was confirmed using primers CBSV10 and 11 (Monger *et al.,* 2001a), and CMBs using Deng primers (Deng *et al*., 1994; Maruthi *et al*., 2002) (PCR methodologies explained below in sections 3.2.7, 3.2.8 and 3.2.9). Plants without any symptoms and free of viruses were further propagated through the micro propagation of nodal buds using tissue culture (TC) techniques as described below (section 3.2.1, 3.2.2 and 3.2.3).

### 3.2. UCBSV and CBSV isolates

Six UCBSV and CBSV isolates (Patil *et al*., 2010) were used in the study, which were collected as stem cuttings (Table 3.1) of unknown cassava varieties in farmers fields (Figure 3.1).

Table 3.1: Six UCBSV and CBSV isolates used in this study.

| Virus isolates[a] | Place collected | Country | Collection date | Collector |
|---|---|---|---|---|
| UCBSV-[UG:Kab4-3:07] | Kabanyolo | Uganda | 2007 | R. W. Gibson |
| UCBSV-[KE:Mwa16-2:08] | Mwalumba | Kenya | 2008 | M. N. Maruthi |
| UCBSV-[TZ:Kib10-2:03] | Kibaha | Tanzania | 2003 | M. N. Maruthi |
| CBSV-[TZ:Nal3-1:07] | Naliendele | Tanzania | 2007 | R. J. Hillocks |
| CBSV-[TZ:Zan6-2:08] | Zanzibar | Tanzania | 2008 | M. N. Maruthi |
| CBSV-[MZ:Nam1-1:07] | Nampula | Mozambique | 2007 | R. J. Hillocks |

[a]Isolates were described by Patil *et al*. (2010).

Plants were grown in NRI's quarantine glasshouse and observed for symptom expression. All the isolates expressed typical but varying CBSD foliar symptoms. Presence of CBSD virus was further confirmed in RT-PCR tests using CBSV 10 and 11 primers (Monger *et al*., 2001a).

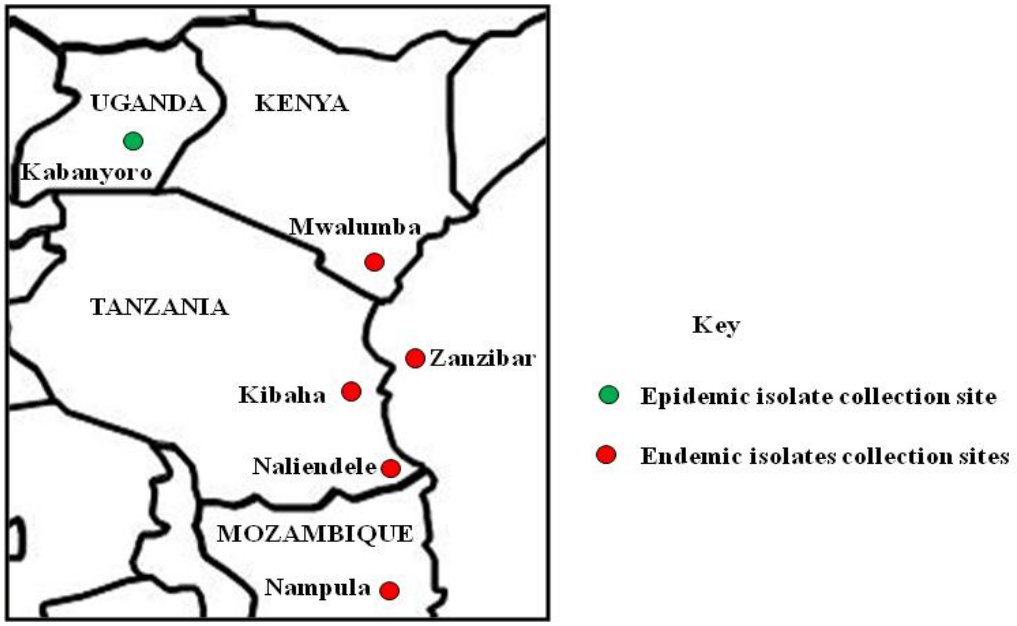



**Figure 3.1**: A sketch map of eastern African countries showing the collection sites of CBSD isolates from the disease epidemic and endemic areas.

### 3.2.1 Media Preparation

The tissue culture method of Frison (1994) was optimised and used in this study for propagation and cleaning experimental plant materials. Basal medium Murashige and Skoog (MS) (Sigma, UK) (Murashige and Skoog, 1962), 2.2 gram (g) and 20 g of sucrose were dissolved in SDW in a beaker, 2 millilitres (ml) of Plant Preservative Mixture (PPM), which is a broad-based and effective pesticide against bacteria and fungi was used. PPM is heat stable and so was autoclaved with media. 50 µl of a growth regulator 1-Naphthaleneacetic acid (NAA) (Thomas, 2006) were added to enhance rooting. The volume was adjusted to 1 L and the pH adjusted to 5.8. Phytagel (Sigma UK) 2 g was added to the solution and dissolved. The media was boiled and 10 ml was dispensed into 25 ml glass tubes (Sterilin, UK). Tubes were closed with plastic caps and autoclaved for 15 minutes (min) at 115 $^{o}$C /15 pound per square inch pressure (PSI). All tools, tubes, and media bottles were wrapped in aluminium foil, and autoclaved (15 min, 121$^{o}$C) as described by Chandler and Haque (1984). A few bottles

containing distilled water were also autoclaved. The laminar airflow cabinet (Esco, UK) was surface sterilised under UV light for 10 min before use. The bench was cleaned with 100% (v/v) ethanol. The outer surface of each autoclaved tube, bottle, or rack was also each spread with 100% (v/v) ethanol before these were placed in the sterile laminar airflow cabinet.

### 3.2.2 Surface sterilizations and inoculation of nodes into the media

Young succulent shoots were selected from cassava plants and cut into small pieces of 1 cm long having at least one nodal bud. The cuttings were washed with running water and sterilized with 70% ethanol for 3-5 sec. The cuttings were transferred into the 10% (v/v) sodium hypochlorite (bleach) and 2-drops of Tween-20 and sterilised by vigorous shaking for 30 min. The cuttings were then washed in sterile SDW 3-4 times until no foam was left in the jar. Using sterile conditions, node cuttings were excised 0.4-0.8 cm in length and transferred into sterile tubes containing MS basal medium. The tubes were covered with sterile plastic lids, labelled and put in the TC growth room for 4-6 weeks under constant environment at 25 ± 2 $^{o}$C, RH 60% and 12 h of light (L12): 12 h of darkness (D12).

### 3.2.3 Transfer of plantlets to the soil

After 4-6 weeks, plantlets were removed from the glass tubes, treated with a systemic fungicide, 0.1% Carbendazim solution (Bayer garden, UK) before planting into plastic pots containing John Innes No. 2 compost. Pots were soaked with a *Bacillus thuringiensis* (Bt)-based biological insecticide Gnat-Off (Hydro garden, UK) 1 ml/litre of water following manufacture's instructions for the control of fungus gnat. Plants were moved to the glasshouse and grown under propagator lids for further 2-3 weeks at 28 ± 2 $^{o}$C, RH 50-60%. Plants were slowly hardened for another 1-2 weeks by slowly lifting the lids. Plants were fed with fertilizer Phostrogen (Bayer Garden, UK) fortnightly and grown for a further 8 weeks before being used in experiments. Plants so obtained were tested by RT-PCR (section 3.1) and used as healthy plants in subsequent experiments.

### 3.2.4 Virus transmission by graft-inoculation of cassava varieties

In order to test the efficiency of graft transmission for UCBSV and CBSV, five cassava varieties (vars), Albert, Kiroba, Ebwanateraka, Columbian and TMS60444, were graft-inoculated with CBSD isolates. Scions of about 10 cm in length were collected from CBSV-infected cassava plants of var. Ebwanateraka expressing clear CBSD symptoms. Scions were cut and all the leaves were removed except for the first unopened and second opened leaves, while the buds were left intact. Sharp scalpels were used to make wedge shaped on scions and a 'V' shaped downward cut on one side of the stem of a rootstock. Scion was immediately inserted into freshly cut rootstock plant. The scion and rootstock plants were secured by wrapping gently but tightly with long strips of plastic tape. On each scion 1-2 young leaves were retained to encourage the exchange of water and nutrients, thus virus movement, with the rootstock. To prevent the excessive loss of moisture and drying of scions, they were enclosed in plastic bags with a few punch holes. After two weeks the protective plastic bags were removed and plants were kept in the glasshouse for symptoms observation. The six UCBSV and CBSV isolates described above (section 3.2.) were used for the graft-inoculation experiments. Five plants were inoculated for each virus-variety combination and allowed to grow for six months. All the control plants were grafted with scions from healthy plants.

### 3.2.5 Buffer solutions

**Preparation of cetyltrimethylammonium bromide (CTAB) buffer for nucleic acid extraction:** For 400 ml extraction buffer is 8 g CTAB (2% w/v), 224 ml of 2.5 M $NaCl_2$, 40 ml 100 mM Tris-HCl, pH 8.0, 16 ml of 20 mM EDTA. The solutions were mixed together and made up the final volume of 400 ml and the pH was adjusted to 8.0.

**Preparation of Tris-borate (TBE) buffer**: To prepare 10×TBE 108 g of 0.45 M Tris-borate and 55 g of $H_3BO_3$ (Boric acid) was dissolved into 40 ml of 0.5 M EDTA. The final volume was made to 1 L. The working concentration of the buffer (0.5 l) was prepared by adding 50 ml of 10×TBE into 1 L of autoclaved SDW. All manipulations were carried out under sterile conditions in a laminar flow. Buffers and media were prepared using SDW.

**The sap inoculation buffer prepared as follows:**

**Solution A**: 0.6 M $K_2HPO_4$ was prepared by dissolving 10.45 g of $K_2HPO_4$ in 100 ml of sterile distilled water (SDW).

**Solution B**: 0.6 M $KH_2PO_4$ was prepared by dissolving 8.17 g of $KH_2PO_4$ in 100 ml of SDW.

The potassium phosphate buffer (inoculation buffer) was prepared by mixing 80.2 ml of 0.6 M $K_2HPO_4$ solution with 19.8 ml 0.6 M $KH_2PO_4$ solution. This was diluted to a final volume of 1000 ml to obtain 0.06 M potassium phosphate buffer. Buffer pH was adjusted to 7.4 with HCl and autoclaved. The buffer was used for preparing virus inoculum and sap inoculation.

### 3.2.6 Sterilisation of solutions and equipment

All glass flask, bottle and plastic equipment, including different sizes (0.5 ml, 1 ml and 1.5 ml) of microcentrifuge tubes and pipette tips used in the experiments were sterilised by autoclaving for 15 min at 115 $^{o}$C / 15 PSI. Other glassware, ceramics and metals were soaked in 5% (v/v) sodium hypochlorite (bleach) for a minimum of 1 h, washed with deionised water and baked for 2 h at 180 $^{o}$C. All solutions and media were prepared with deionised water and sterilised by autoclaving. Metal instruments, including tweezers, scissors and scalpels, were sterilised by soaking in 100% (v/v) ethanol and then burning off excess alcohol in a Bunsen flame.

### 3.2.7 RNA extraction

The CTAB protocol described by Lodhi *et al*. (1994) and optimised for cassava viruses (Maruthi *et al*., 2002), was used for the total ribonucleic acid (RNA) extractions. The protocol was described below:

Total RNA was extracted separately from cassava leaves and experimental host plants (*Nicotiana* spp) infected with UCBSV and CBSV. The third, fourth or fifth leaves from the top of the plants were picked for RNA extraction.

About 100 milligram (mg) of CBSD leaf tissue was placed into a thick gauge plastic bag and ground using roller and mixed with 1000 µl of CTAB extraction

buffer (2% w/v, 1.4 M NaCl, 0.2% (v/v) 2- mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0).

About 750 µl of the samples was poured into a 1.5 ml eppendorf tube and the samples were incubated at 60 $^{o}$C for 30 min. Samples were mixed with 750 µl of phenol: chloroform: isoamylalcohol (25:24:1) by vortexing, to remove protein contaminants. Samples were then centrifuged at 13,000 rpm for 10 min. The top aqueous phase was transferred into new 1.5 ml eppendorf tube.

The samples were precipitated by adding 0.6 volumes (300 µl) of cold isopropanol and incubated at -20 $^{o}$C overnight. The samples were further centrifuged at 13,000 rpm at 4 $^{o}$C for 10 min and the supernatants were discarded. The pellets were washed in 0.5 ml 70% ethanol by vortexing and then centrifuged at 13,000 rpm for 5 min. The ethanol was removed and the pellets were vacuum dried for 5 min. The dried pellets were diluted each in 1000 µl 1x TE buffer (10 mM Tris-HCl, pH 8.0) and stored at -20 $^{o}$C.

### 3.2.8 Reverse transcriptase (RT)

For cDNA synthesis of viral RNA, ImProm-II$^{TM}$ Reverse Transcriptase kit was used following the manufacturer's instructions (Promega, UK). Syntheses was performed as master mix one 5 µl (MM1) and master mix two 15 µl (MM2) in a total volume of 20 µl as described in the reaction mixture below (Table 3.2; Table 3.3).

Table 3.2: Master Mix 1 for cDNA synthesis from virus RNA.

| Reagents | × 1 sample (µl) |
|---|---|
| SDW | 1.0 |
| Oligo-dT primer (20 µM) | 1.0 |
| RNA template | 3.0 |
| Total | 5.0 |

MM1 was incubated at 70$^{o}$C for 5 min and quickly chilled in ice

Table 3.3: Master Mix 2 for cDNA synthesis from virus RNA.

| Reagents | × 1 sample (µl) |
|---|---|
| SDW | 7.5 |
| 5x Impromo-TS-buffer | 4.0 |
| MgCl2 (25 mM) | 2.0 |
| dNTPs (25 mM) | 1.0 |
| Impromo-IITMReverseTranscriptase (200 U/µl) | 0.5 |
| Total | 15.0 |

cDNAs were prepared by mixing MM1 and MM2 in which aliquots were placed in 0.5 ml microfuge tubes. The mixtures were then incubated at 25 $^{o}$C (annealing) for 5 min, 40 $^{o}$C (first strand extension) for 60 min and 70 $^{o}$C (reverse transcriptase inactivation) for 15 min. Thus generated cDNAs were ready for use in PCR. The cDNAs amplification was carried out in a thermal cycler Gene Amp® PCR System 9700 (Applied Biosystems, USA).

### 3.2.9 Polymerase chain reactions (PCR)

For PCR amplifications of viral cDNAs, Red hot polymerase kit (Thermo Scientific, UK) was used. PCR reactions in the final volume of 25 µl included the following reaction mixture (Table 3.4).

Table 3.4: Reaction mixture for PCR amplification of viral cDNAs.

| Reagents | × 1 sample (µl) |
|---|---|
| SDW | 15.9 |
| 10×PCR buffer | 2.5 |
| $MgCl_2$ (2.5 mM) | 1.5 |
| dNTPs (2.5 mM) | 2.0 |
| Forward primer (20 µM) | 0.5 |
| Reverse primer (20 µM) | 0.5 |
| Red hot polymerase (5 U/µl) | 0.1 |
| cDNA template | 2.0 |
| Total | 25.0 |

Table 3.5: The temperature profiles and thermal cycling conditions.

| Steps | Temperature (°C) | Time | Number of cycle |
|---|---|---|---|
| Initial denaturation | 94 | 1 min | |
| Final denaturation | 94 | ½ min | |
| Annealing | 52 | ½ min | ×35 cycles |
| Initial extension | 72 | 1 min | |
| Final extention | 72 | 10 min | |

### 3.2.10 Primers used in RT-PCR reactions

The primers designed previously were used in this study to avoid duplication of work and are listed below (Table 3.6).

Table 3.6: Primers used in PCR and RT-PCR reactions for the detection of CMV, UCBSV and CBSV isolates.

| Virus name | Primer name | Primer sequence (5′-3′) | Product size | Reference |
|---|---|---|---|---|
| Geminivirus | Deng A | TAATATTACCKGWKGVCCSC | | Deng *et al*., |
| | Deng B | TGGACYTTRCAWGGBCCTTCACA | 530 bp | 1994 |
| | CBSV10 | ATCAGAATAGTGTGAACTGCTG*G* | | Monger *et al*., |
| CBSV-CP | CBSV11 | ATGCTGGGGTACAGACAAG | 230 bp | 2001a |
| | CBSVF3 | GGARCCRATGTAYAAATTTGC | | Abarshi *et al*., |
| CBSV-CP | CBSVR3 | AGGAGCWGCTARWGCAAA | 283 bp | 2012 |

### 3.2.11 Gel electrophoresis

RT-PCR products were separated electrophoretically on a 1.2% (w/v) agarose (Themo Fisher Scientific, UK). The gel was prepared by dissolving the agarose in 100 ml of 0.5× TBE buffer (0.045 M Tris-borate, 0.5 mM EDTA, pH 8). The agarose-buffer solution was heated in a microwave oven for 3 min and was cooled to ~ 40 °C before pouring into a gel tray that was fitted with a gel comb. The gel was allowed to solidify for 20 min before loading samples. About 15 µl of the sample was mixed with 5 µl of 5× orange G loading dye and loaded into separate wells on the gel. About 5 µl DNA markers (100 or 1000 base pair (bp)

were loaded into each end slots of the gel. Electrophoresis was performed at 80V for ~ 1 h. The gel was stained in 0.5 µg/ml ethidium bromide solution. The gel was observed under UV light (Syngene G: Box).

## CHAPTER 4: The effect of virus diversity on CBSD symptom expression on cassava and herbaceous host plants[a]

### 4.1. Introduction

Prominent CBSD symptoms appear on leaves in varying patterns of chlorosis based on which, Nichols (1950) identified two types of CBSV isolates. Leaf chlorosis appears in a feathery pattern, first along the margins of the secondary veins, later affecting tertiary veins and may develop into chlorotic blotches. Alternatively, the chlorosis may not be clearly associated with the veins but appears in roughly circular patches between the main veins. In advanced stages of the disease, much of the lamina may be affected. On senescing leaves of some varieties, there is an unusual effect of 'symptom reversion' where the previously chlorotic areas immediately surrounding the veins turn into green areas while the rest of the leaf become chlorotic with bright yellow colours (Hillocks and Jennings, 2003). There is considerable variation in the expression of foliar symptoms depending on variety, growing conditions (temperature, rain fall, and altitude), age of the plant and the virus isolate involved in causing the symptoms (Hillocks *et al*., 1996). Some cassava varieties show marked foliar symptoms but without or delayed root symptoms and *vice versa*. Symptoms of the disease become more difficult to recognize in older plants as the leaves with prominent symptoms are lost (Hillocks *et al*., 2002). New leaves produced from these plants often do not show symptoms, especially at high temperatures. Symptoms can also be transient when a period of active growth produces symptom-free tissues (Jennings, 1960b). However, it's difficult to interpret these observations precisely because they have been made in the field situations with varying agro-climatic conditions on cassava varieties with differing virus resistance levels and crop age, and possibly infected with different virus strains, which all singly or in combination, affect symptom expression.

[a]*The work in this Chapter was published in Advances in Virology, see appendix 3*

It is expected that these studies on CBSV diversity will contribute to an improved understanding of CBSD symptoms diversity which is an essential component of CBSD field diagnosis. These studies are also expected to determine if a severe form of virus is associated with the recent outbreaks of the disease in Uganda.

## 4.2. Materials and methods

### 4.2.1 Cassava varieties and CBSD isolates

Five disease-free cassava varieties Ebwanateraka, Albert, Kiroba, Colombian and TMS60444 (section 3.1) were virus-indexed and the symptomless plants were cultivated through the micro-propagation of nodal buds (section 3.2.2). The six virus isolates were; CBSV-[MZ:Nam1-1:07], CBSV-[TZ:Nal3-1:07], CBSV-[TZ:Zan6-2:08], UCBSV-[KE:Mwa16-2:08], UCBSV-[TZ:Kib10-2:03] and UCBSV-[UG:Kab4-3:07] (Patil *et al.,* 2010; Mbanzibwa *et al.,* 2011) (section 3.2) were used in this study for virus-inoculation on cassava varieties for symptom diversity experiments.

### 4.2.2 Graft-inoculation of virus isolates for recording rate of transmission

The graft-inoculation protocol described before (section 3.2.4) was used for the transmission of the six CBSD isolates onto two-month-old healthy cassava plants of the above five varieties (section 4.2.1). Plants were kept in a relatively constant environment at 28 ± 5 $^{o}$C and 50-60% relative humidity (RH) for symptom development. Various parameters were recorded at weekly intervals for determining the rate of graft-transmission of each isolate on cassava varieties. Time taken for symptom expression and development was recorded on graft-inoculated cassava varieties and on plants grown from CBSD-affected cuttings. Symptoms were recorded for a period of 10 weeks. Data obtained were used to estimate UCBSV and CBSV incubation times in each cassava variety. Two plants grafted with healthy scions in each variety per isolates were used as control.

### 4.2.3 CBSD symptom severity

For each virus-variety combination, 10 cuttings of 10 cm were made from graft-inoculated plants (section 4.2.2) and grown in the quarantine glasshouse. A total of 900 cassava plants were examined for the effect of CBSD infection on the

sprouting of cuttings from infected cassava plants. The effect of virus on cassava growing buds, disease symptom diversity and severity on the leaves of cassava plants were recorded at 28 ± 5 $^{o}$C and 50-60% RH. Number of cuttings that sprouted from each cassava variety was recorded to measure the effect of CBSD on sprouting of young cuttings. Leaf symptom severity was scored on 3-month old plants using a five point scale where 1 = no visible CBSD symptoms, 2 = mild foliar symptoms on some leaves, 3 = pronounced foliar symptoms but no die-back, 4 = pronounced foliar symptoms which might include slight die-back of terminal branches, and 5 = severe foliar symptoms and plant die-back (Hahn *et al*., 1989; Hillocks *et al*., 1996). Plants grown from healthy cuttings were scored as control.

### 4.2.4 Sap-inoculation of herbaceous host plants

Sap transmission of CBSV and UCBSV was conducted at the NRI quarantine glasshouse from March to November, 2008. Thirteen herbaceous plant species/varieties were tested for their response to CBSV by sap-inoculations. For each isolate, a cassava leaf showing clear CBSD symptoms was collected and ground separately in 20 ml of the inoculation buffer using a pestle and mortar. The leaf debris was separated from the sap by squeezing through sterile muslin cloth. Fully-open young leaves of herbaceous plants were sprinkled with fine 600 mesh carborundum powder. The viral sap inoculum was picked up using a cotton wool pad and applied gently on the leaf always stroking from petiole to the leaf tip. Virus inoculated leaves were rinsed thoroughly using a jet of water 10 min after the application of sap and the plants were kept at 28±5 $^{o}$C and 50-60% RH for symptom development. Plants inoculated with buffer alone served as controls. Various parameters were recorded at weekly intervals for determining rate of sap-transmission, symptom type, symptom severity and development.

### 4.2.5 Sampling of plant tissues and virus detection by RT-PCR

Leaf samples were collected by taking the third leaf from the top of cassava and herbaceous plants for CBSV detection by RT-PCR. Samples were collected seven days after inoculation and weekly thereafter for up to 24 weeks. Collected samples were stored at -80 $^{o}$C prior to CBSV testing. The CTAB protocol (section 3.2.7) was used for total nucleic acid extractions. The RT-PCR protocols

(sections 3.2.8 and 3.2.9) were used for both cDNA syntheses and PCR product amplification. Samples that produced bands of expected sizes were classified as positive for CBSV.

### 4.2.6 Measuring virus concentration in infected plants

Virus concentrations of the six CBSD isolates were determined by serial dilutions of cDNA from infected leaf samples with SDW. Total nucleic acids were extracted from CBSD-infected cassava leaves of vars. Albert, Kiroba, Colombian, Ebwanateraka and TMS60444 for each of the six CBSD-isolates. cDNAs were prepared on two samples per isolate using the primer OligodT and diluted subsequently $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ folds. RT-PCR was then carried out on diluted cDNAs using virus-specific primers CBSVF3 and CBSVR3 (Abarshi *et al*., 2011). The concentration of virus particles (RNA) was calculated by recording the initial amounts of cDNAs in each sample using the BioPhotometer (Eppendorf, UK).

### 4.2.7 Statistical analyses

The statistical analyses of the data were carried out using R-software (PC-window, 2009 version). The data for symptom severity scores were processed by two-way analysis of variance $p<0.005$ (ANOVA) using the Tukey test to determine the interaction between viruses and varieties. See section appendix for details of the data analysis.

## 4.3 Results

### 4.3.1 CBSD symptom types on cassava

CBSD symptoms in general were highly variable on cassava but there were two consistent patterns associated with particular isolate/species

UCBSV pattern: Irregular concentric yellow patches. Initial symptoms of this pattern appeared as faint yellowing in small patches along the secondary and tertiary veins of the affected leaf which later developed into bright yellow patches of usually irregular to occasionally circular shapes. The yellow patches are vividly defined and restricted to affected areas. They are not uniformly distributed

throughout the leaflet leaving some parts of the leaf without symptoms. As the symptoms developed further, much of the leaf turned bright yellow while some areas remained green before leaf senescence. These symptoms were associated with isolates from Kabanyolo , Kibaha and Mwalumba (Table 3.1), which are infected with UCBSV and individually be referred to in this study as UCBSV-[UG:Kab4-3:07] (for Kabanyolo isolate), UCBSV-[TZ:Kib10-2:03] (for Kibaha isolate) and UCBSV-[KE:Mwa16-2:08] (for Mwalumba isolate) (Figure 4.1a).

CBSV pattern: Severe leaf feathering and uniform vein clearing symptoms: Initial symptoms of this type appeared as faint green spots which later turned into yellow and eventually became necrotic. The spots were distributed throughout the leaf and not necessarily along the veins. This is followed by the development of feathery yellowing along the secondary and tertiary veins. The yellowing of veins is mostly even, spreading throughout the affected leaf which unlike the UCBSV pattern did not develop into concentric bright yellow patches. These are similar to the classical CBSD symptoms commonly described in the literature.

Senescing leaves appeared completely yellow and the feathery pattern occasionally appeared like a 'water colour painting' on older leaves. These symptoms were associated with the isolates from the coastal lowland areas of Zanzibar, Naliendele (both in Tanzania) and Nampula in Mozambique, which are infected with CBSV and individually be referred to as CBSV-[TZ:Zan6-2:08] (for Zanzibar isolate), CBSV-[TZ:Nal3-1:07] (for Naliendele isolate) and CBSV-[MZ:Nam1-1:07] (for Nampula isolate) (Figure 4.1b).

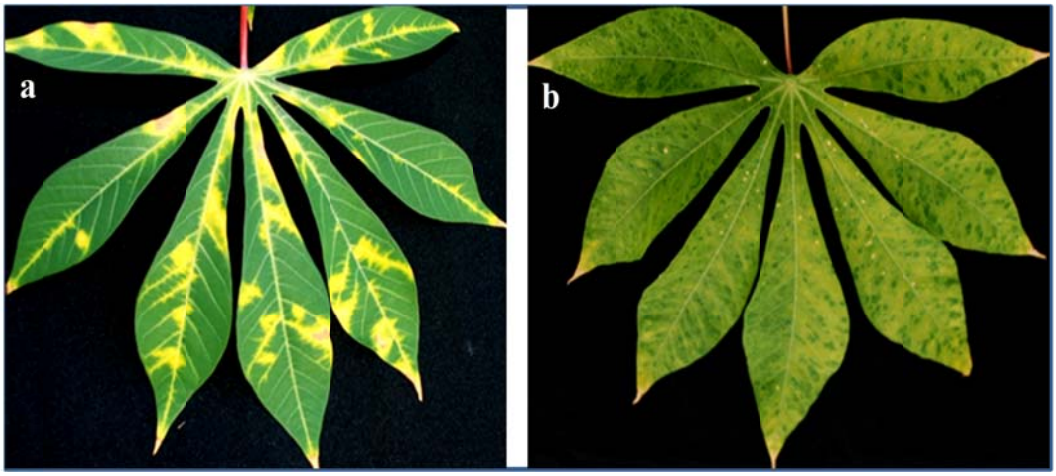



**Figure 4.1**: Cassava leaves (Albert plants) showing symptoms expressed by (a) UCBSV isolates, which are mild irregular concentric yellow patches and (b) CBSV isolates, which are severe leaf feathering and uniform vein clearing symptoms.

### 4.3.2 Rate of transmission of UCBSV and CBSV isolates by graft-inoculation

Plants graft-inoculated with the CBSV-isolates became infected in less than two weeks after grafting and the rates of transmission varied among cassava varieties (Table 4.1). All five cassava varieties graft-inoculated with CBSV expressed symptoms, while only between 2-4 out of five plants graft-inoculated with UCBSV isolates expressed symptoms. The results suggested a smaller UCBSV rate of transmission for grafting compared to CBSV isolates. Development of symptoms on graft-inoculated plants varied between the isolates and for the two virus types. Two CBSV isolates (CBSV-[TZ:Nal3-1:07] and CBSV-[MZ:Nam1-1:07]) infected all plants of the five cassava varieties (Table 4.1) although for the CBSV isolates it was 80-100%. In comparison, the transmission of UCBSV isolates ranged from 60-76%. Amongst the UCBSV isolates; UCBSV-[KE:Mwa16-2:08] produced the greatest percentage transmission (76%). Least transmission was recorded from UCBSV-[UG:Kab4-3:07] (60%)). None of the plants used as control expressed CBSD symptoms (Table 4.1).

Table 4.1: The rate of graft transmission of six CBSUV and CBSV isolates on cassava.

| Cassava variety | Number of plants infected/grafted with each virus isolate[1] | | | | | | Total number of infected/ grafted plants[2] (%) |
|---|---|---|---|---|---|---|---|
| | UCBSV- | | | CBSV- | | | |
| | [UG:Kab4-3:07] | [KE:Mwa16-2:08] | [TZ:Kib10-2:03] | [TZ:Zan6-2:08] | [MZ:Nam1-1:07] | [TZ:Nal3-1:07] | |
| Albert | 4/5 | 3/5 | 4/5 | 4/5 | 5/5 | 5/5 | 25/30 (83) |
| Kiroba | 3/5 | 4/5 | 4/5 | 5/5 | 5/5 | 5/5 | 26/30 (87) |
| Ebwanateraka | 3/5 | 4/5 | 3/5 | 3/5 | 5/5 | 5/5 | 23/30 (77) |
| Colombian | 3/5 | 4/5 | 3/5 | 4/5 | 5/5 | 5/5 | 24/30 (80) |
| TMS 60444 | 2/5 | 4/5 | 3/5 | 4/5 | 5/5 | 5/5 | 23/30 (77) |
| infected/grafted plants[3] (%) | 15/25 (60) | 19/25 (76) | 17/25 (68) | 20/25 (80) | 25/25 (100) | 25/25 (100) | 121/150 (81) |
| Control | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/60 (0) |

[1]Plants were tested by RT-PCR six months after graft-inoculation
[2]Number of infected plants for each cassava variety.
[3]Number of infected plants for each virus isolate.

### 4.3.3 Sprouting of the CBSD-infected cuttings

CBSD-infected cuttings sprouting were recorded in all five cassava varieties at three months after planting. Amongst the isolates, maximum number of cuttings sprouted from the epidemic isolate UCBSV-[UG:Kab4-3:07] (96%) and the least number of cuttings from CBSV-[TZ:Nal3-1:07] (74%) (Table 4.2). Death of plants due to CBSV started within one month of sprouting. In TMS60444, by the end of three months after sprouting, more than half of the CBSV-affected plants had failed to sprout. A limited number of plants failed to sprout in variety Kiroba and Colombian in the UCBSV-infected cuttings (Table 4.2). UCBSV had less severity effect on the cassava growing plants compared to CBSV. All plants used as control have sprouted and none expressed CBSD symptoms.

Table 4.2: The effects of CBSD infections on the sprouting of cassava stem cuttings three months after planting

| Cassava variety | Number of CBSD-infected cuttings that sprouted/planted[3] | | | | | | Total number of sprouted/ planted cuttings[1] (%) |
|---|---|---|---|---|---|---|---|
| | UCBSV- | | | CBSV- | | | |
| | [UG:Kab4-3:07] | [KE:Mwa16-2:08] | [TZ:Kib10-2:03] | [TZ:Zan6-2:08] | [MZ:Nam1-1:07] | [TZ:Nal3-1:07] | |
| Albert | 9/10 | 9/10 | 8/10 | 9/10 | 9/10 | 9/10 | 53/60 (88) |
| Kiroba | 10/10 | 8/10 | 10/10 | 9/10 | 8/10 | 6/10 | 51/60 (85) |
| Ebwanateraka | 10/10 | 7/10 | 10/10 | 8/10 | 9/10 | 10/10 | 54/60 (90) |
| Colombian | 10/10 | 10/10 | 8/10 | 10/10 | 9/10 | 10/10 | 57/60 (95) |
| TMS 60444 | 9/10 | 10/10 | 10/10 | 5/10 | 4/10 | 2/10 | 40/60 (67) |
| Total number of sprouted/ planted cuttings[2] (%) | 48/50 (96) | 44/50 (88) | 46/50 (92) | 41/50 (82) | 39/50 (78) | 37/50 (74) | 255/300 (85) |
| Control[4] | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 60/60 (100) |

[1]Number of sprouted and fully grown plants for each cassava variety.
[2]Number of sprouted and fully grown plants for each virus isolate.
[3]All 10 cuttings were obtained from plants infected with viruses and showing typical CBSD symptoms. Sprouting was recorded at three months after planting.
[4]All the cuttings used as control were obtained from CBSD-free plants

### 4.3.4 CBSD leaf symptoms severity on cassava varieties

Amongst the varieties, the greatest mean severity score was observed on TMS60444 (score 3.1), followed by Ebwanateraka, Albert and Colombian (3.0) while Kiroba had the lowest mean severity score (2.3) (Table 4.3). Amongst the isolates, CBSV-[MZ:Nam1-1:07] was the severest (score 3.8), followed by CBSV-[TZ:Nal3-1:07] (3.7) and CBSV-[TZ:Zan6-2:08] (3.0). UCBSV isolates were least severe with scores ranging from 1.9 to 2.7 (Table 4.3). The leaf symptom severity score for each variety varied (Figure 4.2). When a multiple comparison using two-way analysis of variance (ANOVA), significant differences among cassava varieties were observed for the severity of CBSD symptoms on leaves ($P < 0.001$), virus isolates ($P < 0.001$) and variety versus isolates interactions ($P<0.034$), indicating that some varieties were differentially affected by certain isolates. Plants affected by UCBSV takes long time with no symptoms of CBSD while plants affected by CBSV always developed symptoms from the beginning of sprouting (Table 4.3; Figure 4.2). All plants that sprouted from CBSD-affected cuttings but not showing symptoms have average symptom severity scores of only 1. None of the plants used as control expressed CBSD symptoms (Figure 4.2).

Table 4.3: Mean symptom severity scores for each CBSD isolate on different cassava varieties (on a 1-5 scale using the procedure of Hillocks *et al*., 1996).

| Cassava variety | Mean symptom severity scores for each virus isolate | | | | | | Mean symptom severity[1] |
|---|---|---|---|---|---|---|---|
| | UCBSV- | | | CBSV- | | | |
| | [UG:Kab4-3:07] | [KE:Mwa1 6-2:08] | [TZ:Kib 10-2:03] | [TZ:Zan 6-2:08] | [MZ:Nam 1-1:07] | [TZ:Nal 3-1:07] | |
| Albert | 1.9 | 2.9 | 2.2 | 2.8 | 4.0 | 3.9 | 3.0 |
| Kiroba | 1.9 | 2.0 | 2.0 | 2.4 | 3.0 | 2.7 | 2.3 |
| Ebwanateraka | 1.9 | 2.6 | 2.1 | 2.8 | 4.0 | 4.0 | 3.0 |
| Colombian | 1.9 | 2.9 | 2.1 | 2.9 | 4.0 | 4.0 | 3.0 |
| TMS 60444 | 2.1 | 2.9 | 2.7 | 3.1 | 4.0 | 4.0 | 3.1 |
| Mean symptom severity[2] | 1.9 | 2.7 | 2.2 | 3.0 | 3.8 | 3.7 | 2.8 |

[1]Mean symptom severity for each variety.
[2]Mean symptom severity for each virus isolate.
Plants were scored for symptom severity at six months after sprouting.

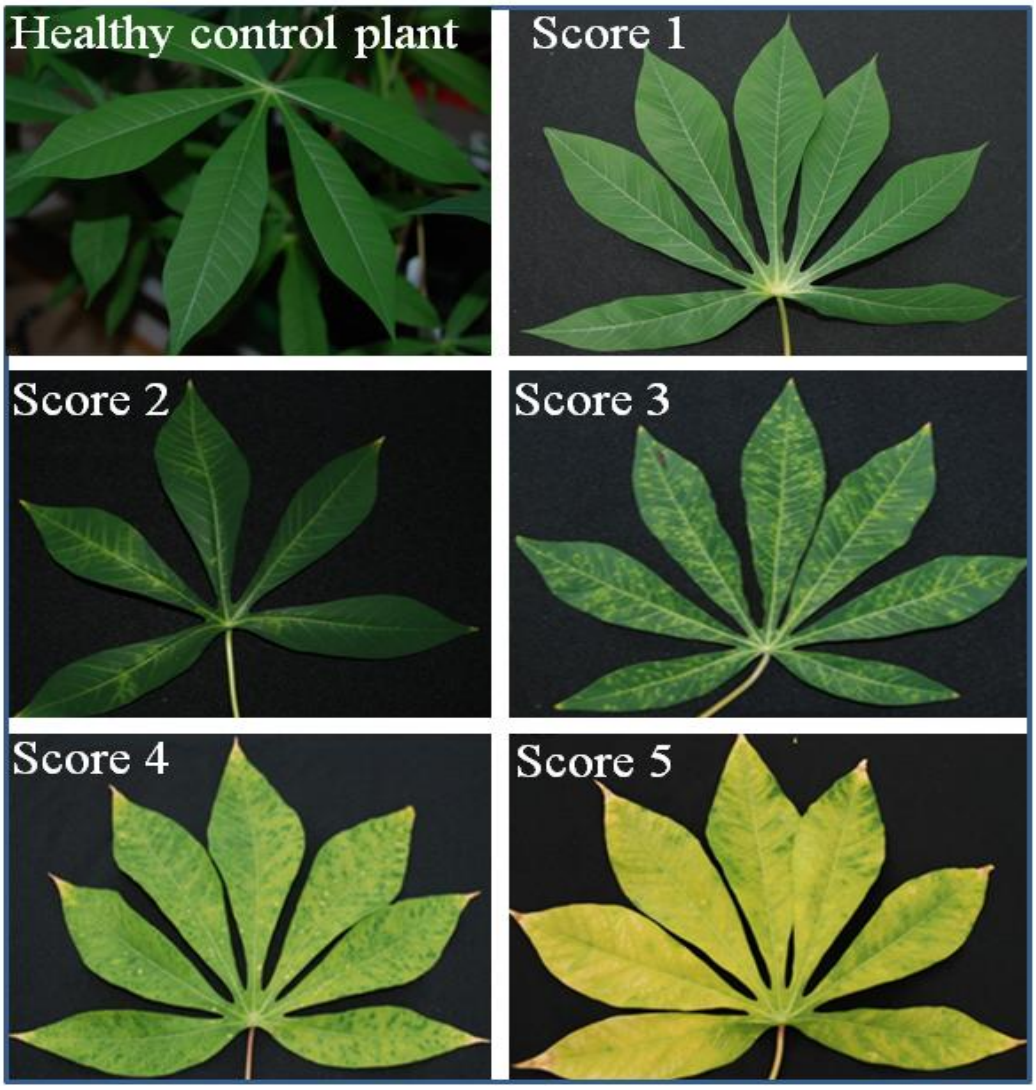



**Figure 4.2**: CBSD symptoms on leaves of affected cassava plants. Plants were visually assessed for development of symptoms at six months after graft-inoculation with UCBSV and CBSV infectious scions. Each plant was scored on a scale of 1–5 where score 1 = symptomless, 2 = mild foliar symptoms on leaves and stems, 3 = pronounced foliar symptoms on leaves, but no die back, 4 = pronounced foliar symptoms on leaves, might or not include die back, 5 = pronounced foliar symptoms including severe die back.

### 4.3.5 CBSD symptom development on cassava varieties

Time taken for the development of CBSD symptoms were recorded on Albert infected with UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. From the first week of symptom appearance, a diseased leaf lasted for about 8-12 weeks after first symptom appearance before dropping off (Figure 4.3). Ninety percent of the leaves dropped within three months of first appearance of the symptoms. For CBSV, symptoms first appeared within two weeks after the graft-inoculation and three weeks for UCBSV except in Kiroba where first symptoms were observed five and six weeks after inoculation, respectively. In plants grown from infected cuttings, symptoms developed on first leaves in weeks 1-2 for both CBSV and UCBSV isolates (Table 4.4). Generally, it took between 3-8 weeks for plants infected with UCBSV and CBSV to attain 100% infection.

Table 4.4: Time taken to express symptoms on CBSD-infected cuttings and graft-inoculated plants in the glasshouse.

| Cassava variety | First/ last symptoms (in weeks) expressed by UCBSV and CBSV isolates[a] | | | | | |
|---|---|---|---|---|---|---|
| | UCBSV- | | | | | |
| | [UG:Kab4-3:07] | | [KE:Mwa16-2:08] | | [TZ:Kib10-2:03] | |
| | cutting | grafted | cutting | grafted | cutting | grafted |
| Albert | 2/5 | 4/4 | 2/5 | 3/5 | 2/5 | 3/6 |
| Kiroba | 4/8 | 6/9 | 3/8 | 6/8 | 4/7 | 6/8 |
| Ebwanateraka | 1/4 | 4/4 | 1/4 | 3/5 | 1/3 | 3/5 |
| Columbian | 2/4 | 4/5 | 2/4 | 3/6 | 2/3 | 3/4 |
| TMS60444 | 2/4 | 4/5 | 1/4 | 3/5 | 2/3 | 3/4 |
| | CBSV- | | | | | |
| | [TZ:Zan6-2:08] | | [MZ:Nam1-1:07] | | [TZ:Nal3-1:07] | |
| | cutting | grafted | cutting | grafted | cutting | grafted |
| Albert | 2/4 | 3/5 | 1/2 | 2/4 | 2/3 | 2/4 |
| Kiroba | 3/6 | 5/7 | 2/4 | 5/7 | 2/4 | 5/5 |
| Ebwanateraka | 1/5 | 2/6 | 1/2 | 3/5 | 1/2 | 2/6 |
| Columbian | 2/4 | 3/7 | 1/5 | 2/4 | 2/3 | 2/4 |
| TMS60444 | 1/3 | 2/5 | 1/1 | 2/2 | 1/2 | 2/2 |

[a]Indicates time to the first symptom appearance/time when all the plants showing symptoms were recorded in weeks.

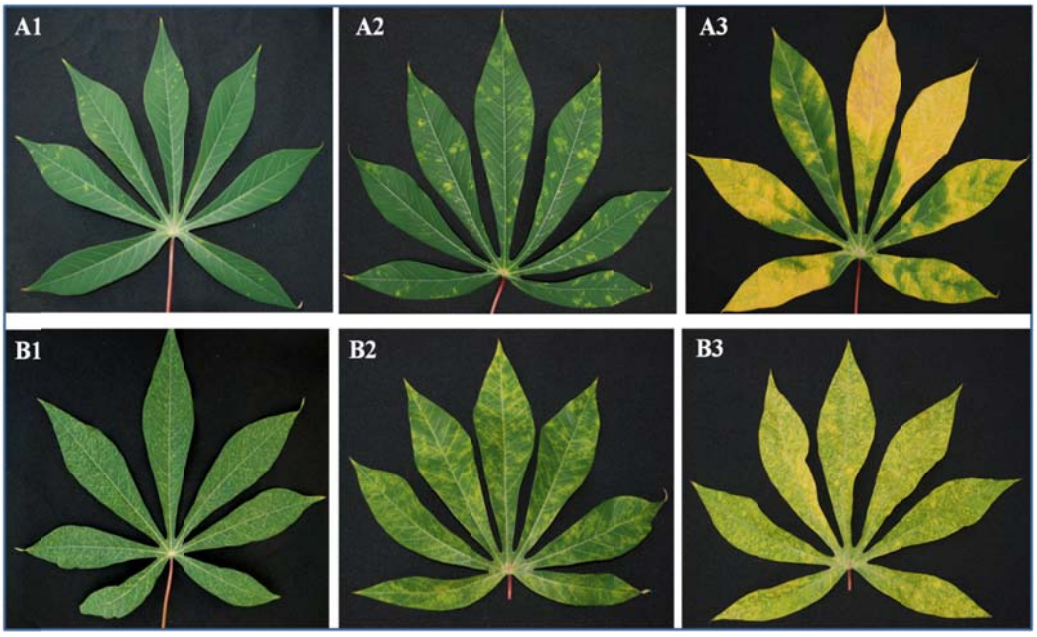



**Figure 4.3:** Typical CBSD symptom development from one month to three months on cassava variety Albert upon infection by UCBSV-[UG:Kab4-3:07] (A1 = initial UCBSV-[UG:Kab4-3:07] symptoms observed in the first month, A2 = symptom development in the second month and A3 = UCBSV-[UG:Kab4-3:07] symptom at three months) and CBSV-[MZ:Nam1-1:07] isolates (B1 = initial CBSV-[MZ:Nam1-1:07]symptom in the first month, B2 = symptom development in the second month and B3 = CBSV-[MZ:Nam1-1:07] symptom at three months).

### 4.3.6 CBSD symptom severity on herbaceous host plants

All six CBSD isolates infected *Datura stramonium*, *Nicotiana clevelandii*, *N. benthamiana N. glutinosa, N. tabacum* nn, *N. tabacum* NN and *N. rustica* with varying rates of infection (Table 4.5). All plants of *N. clevelandii* were infected with each isolate. Most but not all plants of *N. tabacum* nn, *N. tabacum* NN and *N. rustica* were also infected with each isolate.

Symptom severity on herbaceous host plants varied especially on *N. clevelandii* and *N. benthamiana*. Plants infected with CBSV-[TZ:Nal3-1:07] and CBSV-[MZ:Nam1-1:07] were severely stunted and subsequently wilted by developing leaf necrosis (Figures 4.4; 4.5). Most of these plants died usually within four weeks of virus inoculation. Plants infected with the remaining isolates developed various patterns of chlorosis, vein clearing, leaf malformation and stunting but not necrosis and death. Symptoms on other hosts also varied but in general included leaf chlorosis, mosaic and mottling. Local lesions were seen on *N. tabacum* nn, chlorosis/ mosaic patterns in *N. tabacum* NN and vein clearing in *N. benthamiana* by all the isolates. All herbaceous host plants infected with UCBSV and CBSV expressed varying symptoms except *N. tabacum* nn, which expressed local lesions only (Appendix 1.1).

Time taken for first symptom expression on these hosts varied for each isolate and it depended on the virus and plant species infected. Amongst the isolates, CBSV-[MZ:Nam1-1:07] produced symptoms in all hosts within a week of inoculation, which is closely followed by CBSV-[TZ:Nal3-1:07]. Symptom expression ranged from week 1-4 for the remaining five isolates. Of the plant species, *N. clevelandii* was most susceptible, showing symptoms on all plants between weeks 1-3. About 3-7 weeks were required to attain 100% incidence in all the infected *N. benthamiana* and *N. clevelandii* inoculated with CBSV isolates compared to the 3-8 weeks for the UCBSV isolates (Appendix 1.2). None of the plants used as control expressed CBSD symptoms (Figure 4.5).

Table 4.5: Herbaceous hosts inoculated with CBSV and UCBSV isolates

| Species/variety | Number of plants infected/ inoculated for each isolate | | | | | | |
|---|---|---|---|---|---|---|---|
| | UCBSV- | | | CBSV- | | | |
| | [UG:Kab4-3:07] | [KE:Mwa16-2:08] | [TZ:Kib10-2:03] | [TZ:Zan6-2:08] | [MZ:Nam1-1:07] | [TZ:Nal3-1:07] | Mean |
| *C. quinoa* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *C. maxima* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *Datura metel* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *D. stramonium* | 4/10 | 2/10 | 2/10 | 3/10 | 9/10 | 4/10 | 4/10 |
| *Solanum lycopersicum* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *I. batatas* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *N. benthamiana* | 40/40 | 5/40 | 40/40 | 20/40 | 40/40 | 40/40 | 31/40 |
| *N. clevelendii* | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| *N. glutinosa* | 20/40 | 13/40 | 23/40 | 12/40 | 37/40 | 40/40 | 24/40 |
| *N. hesperis* | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |
| *N. tabacum* nn | 19/20 | 17/20 | 20/20 | 20/20 | 20/20 | 20/20 | 19/20 |
| *N. tabacum* NN | 10/10 | 10/10 | 10/10 | 7/10 | 9/10 | 10/10 | 9/10 |
| *N. rustica* | 18/20 | 17/20 | 15/20 | 20/20 | 20/20 | 20/20 | 18/20 |
| Positive /inoculated | 121/210 (58%) | 74/210 (35%) | 120/210 (57%) | 92/210 (44%) | 145/210 (69%) | 144/210 (69%) | 116/210 (55%) |

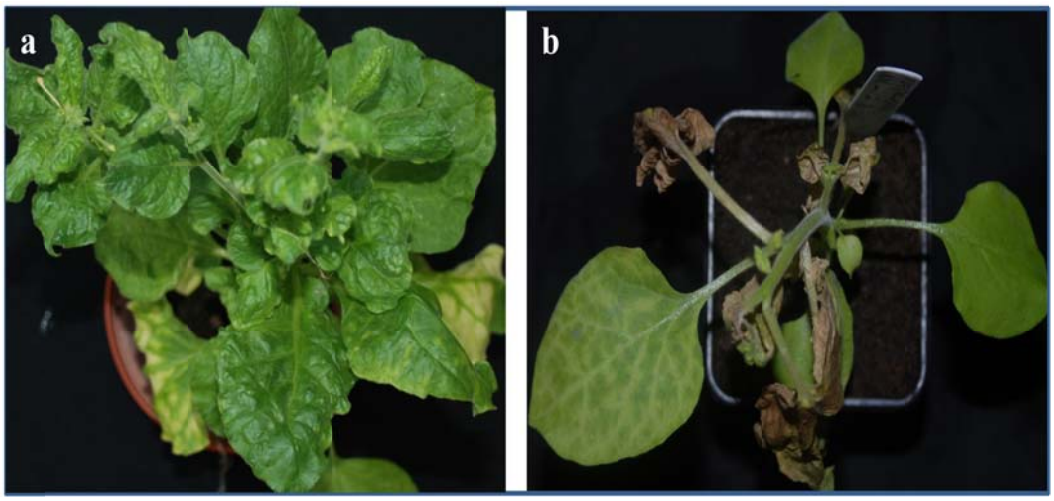



**Figure 4.4:** CBSD-symptoms on *N. benthamiana* showing (a) mild UCBSV symptom on the newly opened leaves in the form of mosaic and (b) severe CBSV symptoms in the form of necrosis, twisting of leaves and stunting of the infected plant.


**Figure 4.5**: Typical symptoms observed on *N. clevelandii* plants 5-6 weeks after inoculated with sap extracted from infected cassava plants. 1 = CBSV-[MZ:Nam1-1:07]; 2 = CBSV-[TZ:Nal3-1:07]; 3 = CBSV-[TZ:Kib10-2:03]; 4 = CBSV-[TZ:Zan6-2:08]; 5 = UCBSV-[KE:Mwa16-2:08]; 6 = UCBSV-[UG: Kab4-3:07]; 7 = Healthy control plant.

### 4.3.7 RT-PCR detection of UCBSV and CBSV isolates

CBSD-infected leaf tissues of cassava plants and herbaceous host plants were tested for the presence of UCBSV and CBSV using primers CBSV F3 and CBSV R3. These primers produced expected RT-PCR fragments of sizes 283 bp. All isolates were readily detected in RT-PCR from *N. benthamiana* (Figure 4.6a) and from cassava samples (Figure 4.6b).

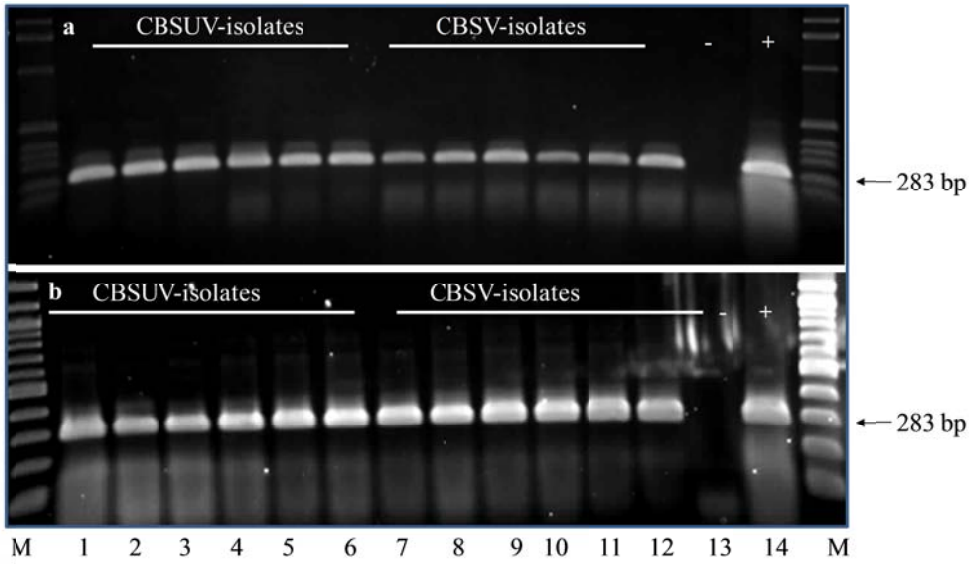



**Figure 4.6:** Detection of UCBSV and CBSV using CBSV F3 and CBSV R3 primers from (a) herbaceous host plants (*N. benthamiana*) and cassava samples (b) (Albert). Lanes 1 and 2 = UCBSV-[UG:Kab4-3:07], 3 and 4= UCBSV-[TZ:Kib10-2:03], 5 and 6 = UCBSV-[KE:Mwa16-2:08], 7 and 8 = CBSV-[TZ:Zan6-2:08], 9 and 10 = CBSV-[MZ:Nam1-1:07], 11 and 12 = CBSV-[TZ:Nal3-1:07], 13 = water control and 14= a known RNA control. The size ladder (M) at each border of the gels is the 100 bp molecular weight marker (New England Biolabs).

### 4.3.8 Measuring virus concentration in infected plants

Relative virus concentrations in a serial dilution of viral cDNA from $10^{-1}$ to $10^{-5}$ fold indicated that virus was detectable at $10^{-5}$ dilutions only from the severe CBSV-[MZ:Nam1-1:07], UCBSV-isolates were not detectable at $10^{-4}$ or above dilutions (Figure 4.7). UCBSV and CBSV isolates were easily detectable at dilutions of up to $10^{-2}$ and $10^{-3}$, UCBSV-isolates produced fainter bands than CBSV.

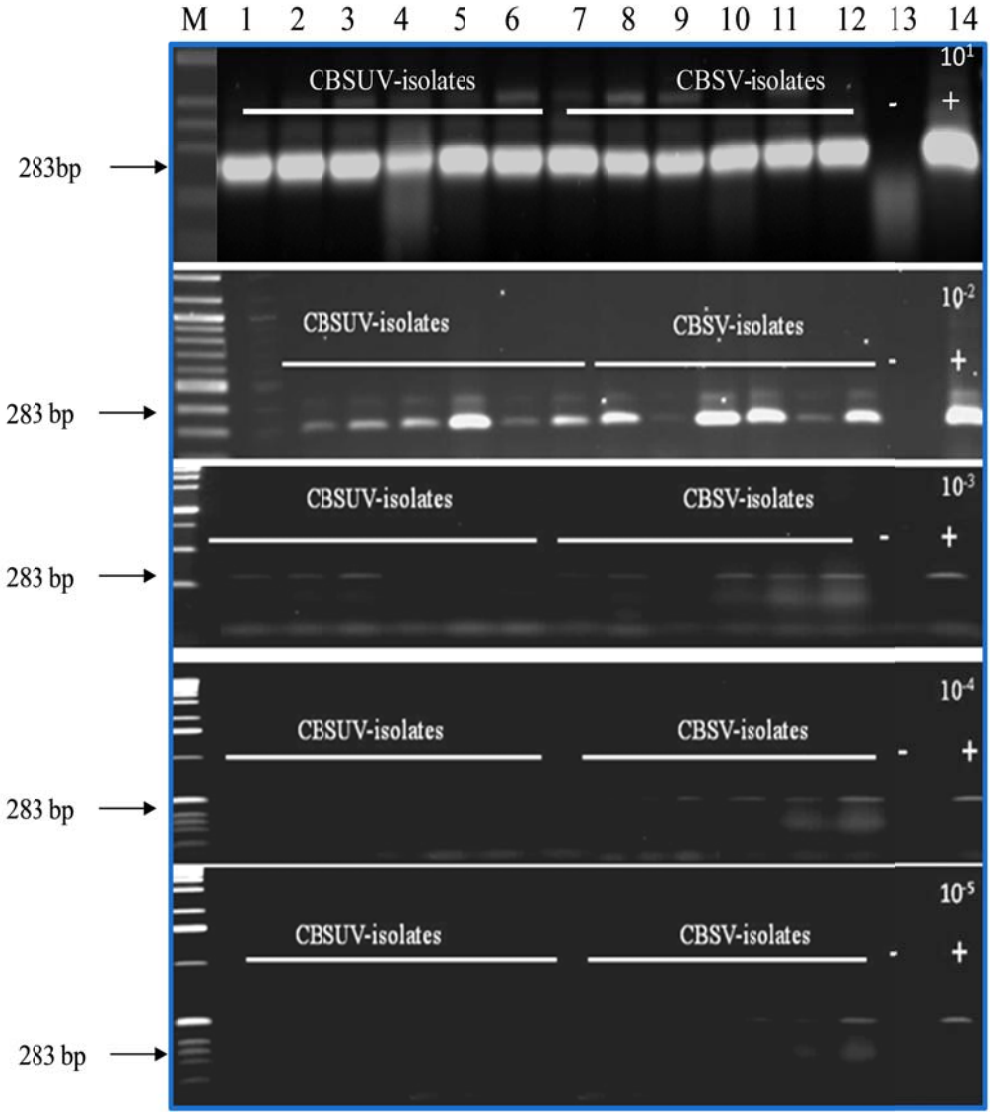



**Figure 4.7**: Detection of UCBSV and CBSV in serial dilutions of cDNA from $10^{-1}$ to $10^{-5}$ folds using CBSV F3 and CBSV R3 primers. Lane 1 and 2 = UCBSV-[UG:Kab4-3:07], 3 and 4= UCBSV-[TZ:Kib10-2:03], 5 and 6 = UCBSV-[KE:Mwa16-2:08], 7 and 8 = CBSV-[TZ:Zan6-2:08], 9 and 10 = CBSV-[MZ:Nam1-1:07], 11 and 12 = CBSV-[TZ:Nal3-1:07], 13 (-) = water control and 14 (+) = a known RNA control. The size ladder at left border of the gels (M) is the 100 bp molecular weight marker (New England Biolabs, UK).

### 4.4 Discussion

Until recently, research on CBSD diversity/severity has largely been restricted to observations in the field on cassava plants of different age, genetic make up and grown in different agro-ecological zones with varying environmental conditions and possibly infected with different virus strains, all of which can singly or in combination, influence symptom development. This made the comparison of the field observations between the various studies particularly difficult and the question of whether a severe form of CBSD is associated with the latest epidemic in Uganda has remained unanswered. Inoculation of herbaceous host plants by various researchers provided somewhat uniform conditions for symptom diversity studies (Bock, 1994) but until recently no such comparison has been made with isolates from the coastal endemic and inland epidemic areas involving the two different species of CBSVs (Mbanzibwa *et al*., 2009; Winter *et al*., 2010). It was particularly difficult to conclude whether the severe CBSD symptoms observed in the fields of coastal Mozambique and Tanzania (Hillocks *et al*., 1996), for example, or the relatively milder leaf symptoms seen in Uganda (severity score of 2.0, Alicai *et al*., 2007) were due to the effect of virus isolate or the tolerance/susceptibility of the cassava varieties being grown in those regions. In this study these external variations were eliminated by carrying out experiments in controlled environmental conditions in a glasshouse and on a standard range of CBSD isolates from both the endemic and epidemic regions to determine if indeed virus from one region was more virulent than others. This was particularly relevant to understand if the new outbreaks of CBSD at high altitudes in Uganda and the Lake Zone areas of Tanzania were due to the development of a severe form of the virus, similar to those observed during the course of CMD pandemic in Uganda in the early 1990s.

In order to investigate this, a number of parameters were used to assess the severity levels between one epidemic and five endemic CBSD isolates including the symptoms on leaves of five infected cassava varieties, the effect of virus on sprouting of cassava stem cuttings, the rate of graft transmission, virus titres in infected leaves as well as symptom severity on herbaceous host plants. Amongst the isolates examined, the endemic isolates CBSV-[MZ:Nam1-1:07] and CBSV-[TZ:Nal3-1:07] produced the most severe symptoms with mean symptom severity

scores of 3.7-3.8 on a five-point scale (Hillocks *et al*., 1996). In comparison, the epidemic UCBSV-[UG:Kab4-3:07] isolate was the mildest with a mean leaf severity score of 1.9. The severity of CBSVs can also be estimated by their ability to affect the young growing buds of infected cassava plants (Nichols, 1950). Using these earlier observations as cues, the differences in the severity levels of the epidemic and endemic isolates were further demonstrated when a significantly greater number of cuttings failed to sprout from the severe endemic isolates compared to the milder epidemic isolate. Between 22-26% of the cuttings failed to sprout when infected with CBSV-[MZ:Nam1-1:07] or CBSV-[TZ:Nal3-1:07] while only 4% of the cuttings were similarly affected by the infection of UCBSV-[UG:Kab4-3:07]. These observations were further supported by the greater rates of virus transmission by grafting of the endemic severe isolates which is probably due to high virus titre (about 1000-times greater virus titre in the two severe endemic isolates CBSV-[MZ:Nam1-1:07] or CBSV-[TZ:Nal3-1:07] compared to the epidemic isolate UCBSV-[UG:Kab4-3:07]). A notable difference observed between this and earlier studies, however, is the infection of Albert by all isolates of this study. In graft inoculation experiments, Winter *et al*. (2010) failed to infect Albert by the CBSD isolates from Kenya, Uganda and Malawi. While the difference between these two similar studies could not be explained at this stage, these results nonetheless have great implications for developing disease management strategies since Albert once considered being a potential source of resistance to CBSD in Kenya, Uganda and Malawi is now proven susceptible. In southern Tanzania, growing of Albert has been largely abandoned due to its susceptibility to CBSD there (RJ Hillocks, unpublished).

The differences in the symptoms were also observed on infected herbaceous hosts. Compared to the previously reported *N. benthamiana* (Mbanzibwa *et al*., 2009; Winter *et al*., 2010), *N. clevelandii* in particular was highly susceptible to both CBSV and UCBSV in our conditions, and this could be an excellent differential host for separating severe and milder isolates. On *N. clevelandii*, the severe isolates CBSV-[MZ:Nam1-1:07] and CBSV-[TZ:Nal3-1:07] produced symptoms early, caused severe stunting of infected plants, leaf necrosis and often plant death. The remaining isolates including UCBSV-[UG:Kab4-3:07] caused various forms of leaf chlorosis, the symptoms were less severe and non-lethal.

Put together, these collective observations on symptom diversity did not indicate the association of a severe form of CBSD in Uganda. These results are indeed consistent with studies on another epidemic isolate (Namulonge) from Uganda (Winter *et al*., 2010) and especially agree with field observations in which the maximum average severity recorded at the onset of CBSD in Uganda was only 2.0 (Alicai *et al*., 2007). In the absence of a particularly virulent virus in Uganda, our results, however, raise serious questions as to the factors responsible for the current outbreaks of CBSD in eastern African countries. The possible explanations for this are the presence of unusually high populations of whitefly vectors (*B. tabaci*) on cassava that may be responsible for the rapid spread of the virus in the field, the recent widespread introduction of CMD-resistant varieties that are particularly susceptible to CBSD, or the combination of both. Recent surveys in Uganda indeed confirmed these possibilities, where more than 70% of the cassavas grown in 23 districts were CMD-resistant 'improved' varieties, all of which are susceptible to CBSD. These varieties also support high whitefly numbers, in excess of 200 adults for top five leaves (Maruthi MN, personal observations in the field). Although such 'elite' cassava has not been introduced in high quantities to the Lake Zone Tanzania, the high susceptibility of local land races grown in the region and the sudden development of unusually high whitefly populations on cassava there is ensuring the spread of CBSD (Jeremiah and Legg, 2008; Legg *et al*., 2011). Identification of severe forms of CBSVs in CBSD endemic regions is particularly worrying because the spread of these isolates into areas of high whitefly population has greater potential to cause even more severe damage to cassava production than yet encountered. Our results emphasize the need for exercising strict quarantine measures for preventing further spread of CBSD between country borders and have also identified the need for developing cassava varieties with broad spectrum resistance to both viruses.

### 4.4.1 Conclusions

The main conclusions arising from Chapter 4 are:

1. All the five cassava varieties infected with UCBSV-[UG:Kab4-3:07]) isolate produced relatively milder symptoms compared to the same varieties infected with the remaining five isolates.

2. Differences in symptom severity following infection by CBSV isolates and UCBSV isolates is attributed to differences between the virus species and also, to differences between the host varieties.

3. CBSV-[MZ:Nam1-1:07] and CBSV-[TZ:Nal3-1:07] are more pathogenic on *N. benthamiana* and *N. clevelendii* than the remaining four isolates.

# CHAPTER 5: Examining the non-vector modes of transmission of Cassava brown streak viruses

## 5.1 Introduction

The whitefly, *B. tabaci*, was shown to be the vector of cassava brown streak viruses (Maruthi *et al*., 2005; Mwere *et al*., 2009). Recently there was increased spread of CBSD in many areas of East Africa (Alicai *et al.,* 2007; Legg *et al*., 2011) and this has raised additional questions on the mode of CBSV transmission. This was because the low rates of transmission obtained by Maruthi *et al.* (2005) and Mwere *et al*. (2009) in controlled laboratory conditions could not explain the high rates of disease spread in the field. Previous attempts to transmit the virus by other suspected insect vectors such as the aphid, *Myzus persicae* Sulz (Hemiptera: *Aphididae*) were also unsuccessful. The lack of appreciable rate of transmission by vector has brought about suspicion over the contribution of non-vector modes in the spread of the virus. The rate of transmission of virus was not stated clearly in artificial sap-inoculation conducted by Lister (1959), or the efficiency of the method in comparison to other methods. Similarly, graft-inoculation of CBSV described by Storey (1936; 1939) did not report the efficiency of the technique as compared to other methods.

CBSD was also thought to be transmitted naturally between healthy and infected cassava plants the field (Hillocks *et al.,* 1999; Kanju *et al.,* 2003a). Other non-vector methods of virus transmission including contaminated tools, hand leaf picking (a procedure followed in some SSA countries to harvest leaves) and by sap have no t previously been studied. Studies were therefore, undertaken to determine if CBSVs can be transmitted by a) contaminated tools while cutting infected and uninfected plants, (b) leaf picking (c) sap-inoculation of cassava varieties and (d) to compare these to that of virus inoculation by grafting.

## 5.2 Materials and Methods

### 5.2.1 Cassava varieties and UCBSV and CBSV isolates

Two disease-free susceptible cassava varieties Albert and TMS60444 were grown and tested to confirm the absence of virus in them. Albert and TMS60444 were

used because of their susceptibility to both UCBSV and CBSV. UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] were used in the experiments.

### 5.2.2 Sap-inoculation

Sap-inoculation experiments were conducted from February to December, 2008. The protocol followed for sap-inoculation of the healthy plants of vars. Albert and TMS60444 is described in section 4.2.4. Each treatment comprised of 10 plants for each variety-virus combination, and the experiment was repeated three times which contained a total of 120 inoculated plants for two varieties (10 plants x 3 replications x 2 varieties x 2 isolates = 120). The inoculated plants were further grown in the quarantine glasshouse and observed for symptom development for at least three months. Plants inoculated with buffer alone served as controls. The efficiency of transmission of UCBSV and CBSV was determined by assessing the presence of the virus in inoculated plants six months after inoculation. The number of weeks to the first appearance of CBSD leaf symptoms was recorded and plants were tested for virus by RT-PCR.

### 5.2.3 Sap-injection

CBSD-infected sap was collected directly from 10 month old CBSD-infected plants of var. Ebwanateraka (Figure 5.1a) using 10 ml sterile syringe (Plastipak, UK) and 5 mm rubber tube (Smith medical international, UK). Plants were cut at about 1-2 feet from the bottom and the cut end was attached to the rubber tube. The sap that was released from the cut end was collected in the rubber tube, which was collected using a syringe. The collected sap was then injected onto the healthy cassava plants of Albert and TMS 60444 using a needle. Sap was injected at the base of the leaf petiole as this was the soft part of the plant (Figure 5.1b). Ten plants for each variety-virus combination were sap-injected and observed for symptom development. The experiment was replicated thrice, which contained a total of 120 inoculated plants. Plants injected with sap collected from healthy plants served as controls. The efficiency of transmission of UCBSV and CBSV by this method was determined as described above (section 5.2.2).

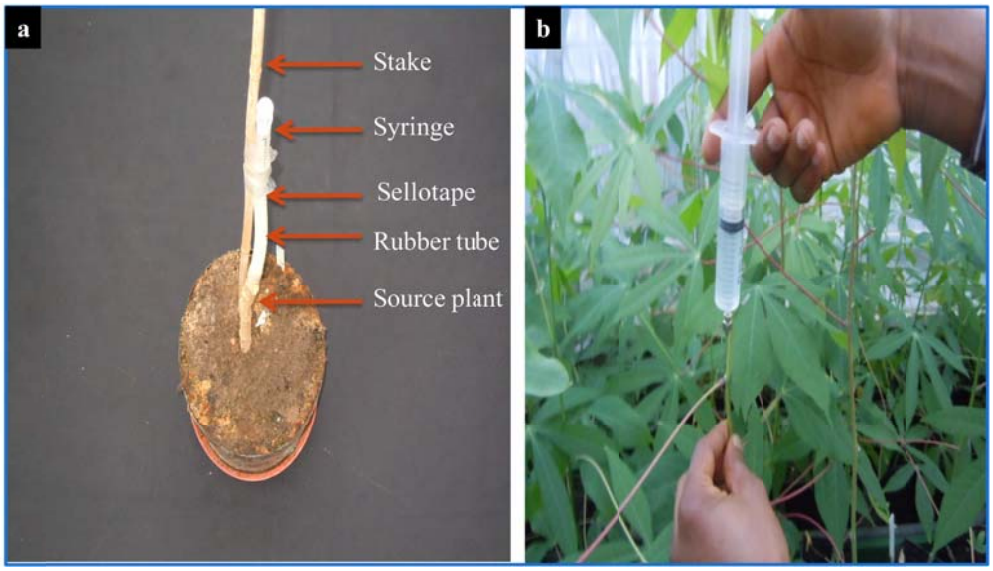



**Figure 5.1**: Collection of CBSD-infected sap (a), injection onto healthy cassava plants (b).

### 5.2.4 Leaf picking

An experiment to test the possibility of virus transmission through leaf picking, which is a process commonly practiced by farmers for leaves as source of food and animal feeds (Figure 5.2), was conducted in the quarantine glasshouse at NRI, from March to December, 2009. Ten plants for each variety Albert and TMS60444 were grown in pots alongside 20 CBSD-infected cuttings of the var. Ebwanatareka. At three months of age, leaves were harvested from CBSD-affected as well as -free plants alternatively with the intention of transmitting the virus by contaminated hands. Leaf picking was done three times for a total of 120 plants for Albert and TMS60444. Experimental plants were kept in the glasshouse and observed for CBSD symptom expression for six months. Leaf picking between the healthy plants served as controls. The efficiency of transmission of UCBSV and CBSV by this method was determined as described above (section 5.2.2).

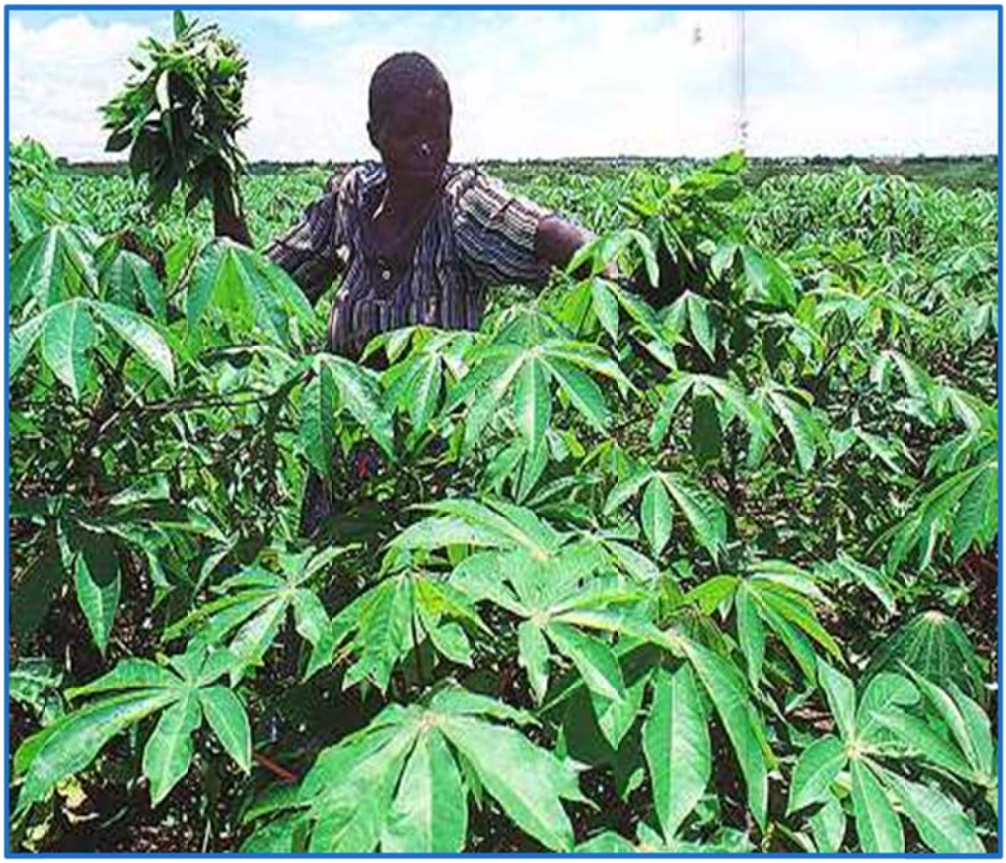


**Figure 5.2**: Leaf picking in the field by a farmer in Tanzania (photo: courtesy of R.J. Hillocks)

### 5.2.5 CBSD-contaminated tools

To assess infected cutting tools (Secateurs) as a potential source of virus spread, experiments were conducted from April to December, 2008. Twenty CBSUV and CBSV-infected cuttings of the var. Ebwanateraka were established individually in pots alongside 50 healthy plants for each Albert and TMS60444. After three months, a pair of secateurs was used to cut an infected stem var. Ebwanateraka. The contaminated secateurs were then used to cut healthy plants (Figure 5.3). A single cut of an infected stem was followed by a cut on a healthy plant. Following this process, 30 plants were inoculated for each variety and virus type (UCBSV and CBSV). The experiment was replicated thrice, which contained a total of 120 inoculated plants. Ten plants of each variety were maintained as control, which were cut using uninfected secateurs. The efficiency of transmission of UCBSV and CBSV by this method was determined as described above (section 5.2.2).

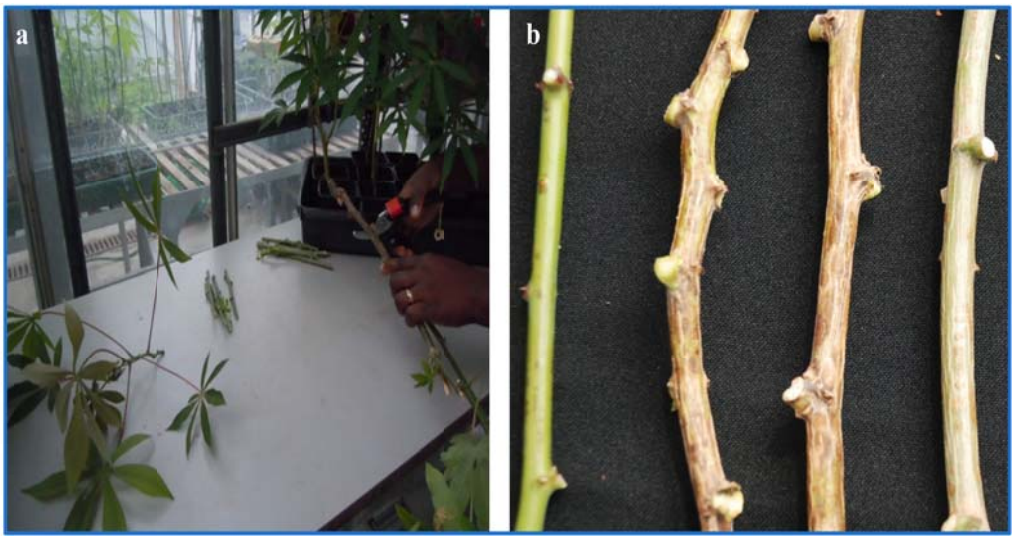



**Figure 5.3**: Transmission of CBSUV and CBSV using infected secateurs (a), CBSD-infected stem cuttings used as virus source for contaminated tools experiment (b).

### 5.2.6 Graft-inoculation

The graft-inoculation protocol described before (section 3.2.5) was followed. Five month old plants of Albert and TMS60444 were graft-inoculated to compare the efficiency of UCBSV and CBSV transmission in comparison with other non-vector transmission methods. The experiment was also repeated three times, and contained a total of 120 graft-inoculated plants for each variety. Ten plants of each variety were graft-inoculated with healthy scions as controls. The efficiency of transmission of UCBSV and CBSV by this method was also determined as described above (section 5.2.2).

## 5.3 Results

### 5.3.1 Sap-inoculation

CBSV-[MZ:Nam1-1:07] was transmitted by sap-inoculation from infected cassava to virus-free cassava varieties but not UCBSV-[UG:Kab4-3:07] (Table 5.1). A period of eight weeks was required before the CBSD symptoms expressed in the sap-inoculated plants. A total of 23% of TMS60444 plants inoculated with CBSV-[MZ:Nam1-1:07] tested positive for the virus compared to 17% for Albert (Figure 5.4).

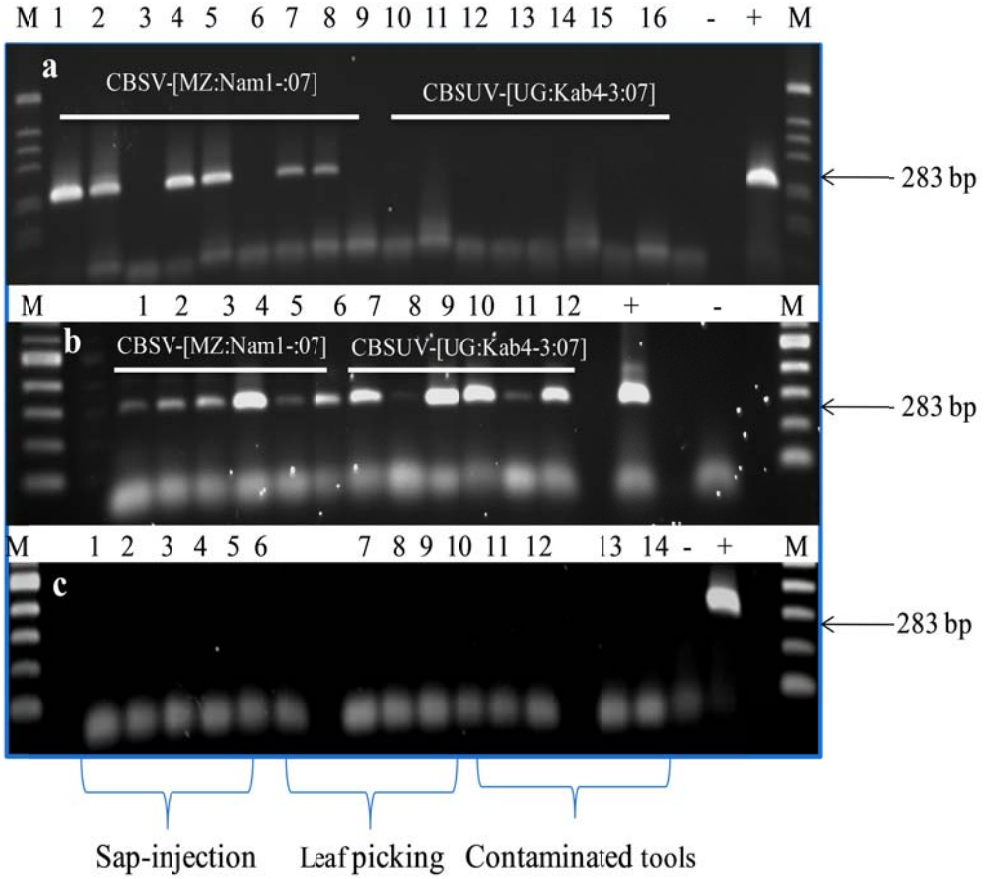



**Figure 5.4**: A representative picture of agarose gel electrophoresis of RT-PCR amplified products for samples from UCBSV and CBSV transmission experiments using CBSV F3 and CBSV R3 primers. (a) Samples tested from sap-inoculation, (b) samples tested from graft-inoculation and (c) for other modes of transmission. (-) negative control from healthy control plants; (+) positive control from CBSD-infected plant. The size ladder (M) at each border of the gels is the 100 bp molecular weight markers (New England biolabs).

### 5.3.2 Sap-injection

None of the plants from both Albert and TMS60444 sap-injected with the two isolates exhibited CBSD symptoms. All plants tested negative in RT-PCR after six months (Figure 5.4; Table 5.1).

### 5.3.3 Leaf picking

Similarly, none of the tested plants from Albert and TMS60444 expressed CBSD symptoms in the leaf picking experiment for the two virus isolates. All plants tested were negative by RT-PCR (Figure 5.4; Table 5.1).

### 5.3.4 CBSD-contaminated tools

None of the cuttings made from virus contaminated secateurs sprouted with CBSD symptoms six months after planting. CBSVs were not detected by RT-PCR (Figure 5.4; Table 5.1).

### 5.3.5 Graft-inoculation

CBSV-[MZ:Nam1-1:07] was transmitted with 100% efficiency to both varieties while the rates of UCBSV-[UG:Kab4-3:07] transmission varied between 77-80% (Table 5.1). The time taken for symptom expression between the viruses also varied. Plants infected with CBSV-[MZ:Nam1-1:07] expressed symptoms in 1-2 weeks, while UCBSV-[UG:Kab4-3:07]-infected plants took 4-5 weeks. All the symptomatic plants were tested positive by RT-PCR (Figure 5.4; Table 5.1) and asymptomatic and control plants tested negative.

Table 5.1: Summary of non-vector modes of transmission of UCBSV and CBSV.

| | Control | | Time to CBSD symptoms (week) | | CBSVs-positive (RT-PCR) | | Efficiency of transmission (%) | |
|---|---|---|---|---|---|---|---|---|
| Treatment | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Sap-inoculation | | | | | | | | |
| Albert | 0/10 | 0/10 | - | 8 | 0/30 | 5/30 | 0 | 17 |
| TMS60444 | 0/10 | 0/10 | - | 7 | 0/30 | 7/30 | 0 | 23 |
| Sap-injection | | | | | | | | |
| Albert | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| TMS60444 | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| Leaf picking | | | | | | | | |
| Albert | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| TMS60444 | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| Contaminated tools | | | | | | | | |
| Albert | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| TMS60444 | 0/10 | 0/10 | - | - | 0/30 | 0/30 | 0 | 0 |
| Graft-inoculation | | | | | | | | |
| Albert | 0/10 | 0/10 | 5 | 2 | 23/30 | 30/30 | 77 | 100 |
| TMS60444 | 0/10 | 0/10 | 4 | 1 | 24/30 | 30/30 | 80 | 100 |

-; indicated no CBSD symptom was observed and no CBSVs detected by RT-PCR at six months after inoculation.

## 5.4 Discussion

The main objective of this study was to determine the efficiency of transmission using non-vector modes of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] to two susceptible cassava varieties. In sap-inoculation experiments, slightly greater transmission rates of CBSV-[MZ:Nam1-1:07] (23%) was achieved on TMS60444 compared to Albert (17%), which probably indicates that TMS60444 is more susceptible to CBSD than Albert, although no differences were observed in symptom expression.

Graft-inoculation was the most efficient and effective of the techniques assessed because up to 100% transmission was attained, notably for the CBSV-[MZ:Nam1-1:07] isolate. UCBSV-[UG:Kab4-3:07], which was not transmitted through sap-inoculation, but 77-80% graft-transmissible. The rate of graft-transmission of UCBSV on TMS60444 obtained in this study was low compared to rate obtained by Yadav *et al*. (2011) (100%). In addition, a relatively short time was required for virus detection and symptom expression when UCBSV-[UG:Kab4-3:07] or CBSV-[MZ:Nam1-1:07]-infected scions were grafted onto healthy cassava plants, which further suggests that this technique is ideal for virus transmission studies. The findings of this study are consistent with CABRI (1998) that graft-inoculation is an efficient way of transmitting viruses that are not readily or not at all transmissible by sap to susceptible host plants. The study further demonstrated that graft-inoculation is achievable for both viruses and at high transmission rates, suggesting the technique is suitably efficient for indexing and detection of UCBSV and CBSV.

Both UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] were not transmitted through hand leaf picking, contaminated tools or sap-injection which contradicts results obtained in other virus studies (Ferguson, 2009; Calvert and Thresh, 2002). These results are not entirely surprising since both infected and healthy cassava plants are cut using single tool both by farmers and researchers, often unknowingly, but incidences of diseases transmitted by contaminated tools have not been known and these techniques may therefore not contribute to the spread of UCBSV and CBSV. However, there are viruses and virus-like particles (viroids) that can be transmitted by contaminated tools, which include *Cassava*

*common mosaic virus* (CsCMV) in cassava, spindle tuber viroid, citrus exocortis viroid in citrus, *Potato virus X* in potato and *Pepino mosaic virus* (Manzer and Merriam, 1961; Broadbent *et al.*, 1968; Calvert and Thresh, 2002; Ferguson, 2009). The findings of this study are promising with regard to the avoidance of non-vector sources of virus transmission in that it is not necessary to sterilize pruning tool in order to prevent transmission of UCBSV and CBSV. This knowledge will be of particular value to farmers and researchers alike; who routinely produce cuttings using single cutting tools, and should reassure users that such practice will not lead to the transmission of UCBSV and CBSV. Of relevance to researchers working on cassava viruses, the lack of transmission of the UCBSV and CBSV through contaminated tools suggests a safe base for *in situ* maintenance and propagation of different CBSD isolates together in one place in the glasshouse which offers a great opportunity for research purposes through the economy of space.

The lack of transmission of the virus through leaf picking suggests that 'normal' agronomic practices including touching the plants and leaf picking/harvesting does not contribute to the spread of UCBSV and CBSV. The inability of both UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] to be spread from infected hand is important not just for disease epidemiology but also for research purposes as keeping two viruses in a laboratory does not result in cross contamination.

### 5.4.1 Conclusions

The main conclusions arising from Chapter 5 are:

1. Both virus isolates produced symptoms in both varieties upon graft-inoculation

2. CBSV and UCBSV are not transmissible from contaminated secateurs, leaf picking between diseased and healthy plants and direct injection of sap collected from CBSD-infected cassava plant.

3. Among all the non-vector transmission techniques tested, graft-inoculation is most efficient for transmitting both UCBSV and CBSV. However, since graft-inoculation is not a routine practice done by farmers and yet the other routine management practice seemed not to contribute to UCBSV and CBSV spread. Whitefly (*B. tabaci*) and use of already cassava-infected materials are responsible for CBSD perpetuation in farmer's field.

## CHAPTER 6: Mechanisms of resistance to CBSD in cassava varieties

### 6.1. Introduction

The symptoms of CBSD on cassava vary, depending largely on the tolerance level of the varieties and the virus type (Hillocks *et al*., 1996). Cassava varieties in the field differ in their symptom expression. Those that show foliar symptoms but in which the expression of root necrosis is delayed or absent are referred to as CBSD 'tolerant' varieties (Hillocks *et al*., 2002; Walkey, 1985). Some varieties show decreased incidence of foliar symptoms and may or may not succumb to root necrosis (Hillocks and Jennings, 2003). The term 'resistance' in this context means that fewer plants become infected or that disease development is restricted after infection. A variety of cassava is considered susceptible if the virus can fully complete three main processes in the host: genome replication, cell to cell movement (local) and long distance (vascular-dependent) movement (Carrington and Whitham, 1998). Symptom expression within a susceptible host may vary depending on virus isolate, environmental conditions and physiological aspects of the host's response to infection. These interactions collectively result in changes in host's physiology, growth and symptoms. Variation in the expression of CBSD among cassava varieties was reported (Hillocks and Jennings, 2003), suggesting that some inherent characteristics of the varieties control resistance/susceptibility.

There are no reported studies on virus-host interactions for CBSD that have investigated the mechanisms of susceptibility or resistance under uniform controlled conditions. There is limited and conflicting information on resistance to CBSD. Due to the limited molecular information on virus–host interactions, especially concerning resistance or susceptibility, experiments were initiated for a greater understanding of the mechanisms of resistance to CBSD by assessing the differences in cassava varieties with respect to (i) symptom expression and virus replication over time (ii) determine the rate of reversion from UCBSV and CBSV infection (iii) determine varietal differences in terms of vector fecundity, reproduction and survival, and (v) to determine the susceptibility of cassava varieties to the viruses by whitefly (*B. tabaci*) transmission.

## 6.2. Materials and methods

### 6.2.1 Cassava varieties and virus isolates

Cassava varieties Albert (CBSD susceptible), Kiroba (tolerant), and Kaleso (field-resistant) were tested for virus as described before (section 3.2.1, 3.2.2 and 3.2.3). Kaleso is a widely adopted CBSD-resistant variety in Kenya, and Kiroba is a widely grown Tanzanian landrace (Hillocks, 2003; 2005; 2006). The two virus isolates that were identified in the symptom diversity study (section 4.3.4); CBSV-[MZ:Nam1-1:07] (severe) and UCBSV-[UG:Kab4-3:07] (relatively mild) were used in experiments to measure virus movement, titre and the rate of reversion. Transmission by the whiteflies was done only with CBSV-[MZ:Nam1-1:07]. The isolates were graft-inoculated on to the healthy cassava plants following the protocol described before (section 3.2.4). The methods for *in vitro* propagation of cassava varieties and sample preparations, RNA extractions using CTAB method, cDNA synthesis and RT-PCR were also described in Chapter 3 (section 3.2.1, 3.2.2, 3.2.3, 3.2.7, 3.2.8 and 3.2.9). Three months after planting (MAP), plants were transferred and grown in relatively large pots (283 mm diameter, which can accommodate 10 litres of compost) to facilitate robust growth and the development of roots for sampling.

### 6.2.2 Grafting of cassava varieties, symptom development and severity

The graft-inoculation protocol described (section 3.2.4) was used for the transmission of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] isolates onto two-month-old healthy cassava plants of the above three cassava varieties (section 6.2.1). Grafting was repeated at four week intervals until all the plants became infected. Plants were kept in a relatively constant environment at 28 ± 5 $^{o}$C and 50-60% RH for symptom development. The efficiency of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] transmission by graft-inoculation, on each variety was calculated as the number of plants with CBSD leaf (chlorosis, vein clearing, and blotches), stem (brown streak or lesion) or root symptoms (necrosis or constrictions) expressed as a percentage of the total number of plants in each variety. CBSD symptoms on leaves were recorded on each variety at four week interval. The severity of symptoms was rated according to the Hillocks *et al*. (1996) scale, as described in section 4.3.4. Symptoms on roots were recorded

96 weeks after graft-inoculation of all the plants by cutting the roots at 1 cm interval. A cut was made from the distal end of each root and photographed (using camera Nikon D5000). The severity of root necrosis was rated by visual inspection on a 5 point scale of 1-5 using the scoring methods of Hillocks *et al*. (2001) and McSween (2006), which was described as: 1 = no visible root discoloration, 2 = presence of small yellow or brown necrosis on the cross sections of the root, 3 = presence of medium (2-10%) brown or black necrosis on the cross section of the root, 4 = presence of severe (10-30%) brown or black necrosis on the cross section of the root, 5 = very severe (>30%) brown or black necrosis on the cross section of the root.

### 6.2.3 Sampling for measuring virus detection and movement in cassava

Twentyfour hours after graft-inoculation, leaves and root samples from three cassava varieties (Kaleso, Kiroba and Albert) were collected and analysed by RT-PCR. Three graft-inoculated plants were selected for sampling from each cassava variety infected with UCBSV-[UG:Kab4-3:07] or CBSV-[MZ:Nam1-1:07]. Samples were taken from leaves (third or fourth leaf from top), secondary and tertiary roots at 24 h intervals in the first week. Subsequently, samples were collected at weekly interval for 4 weeks, followed by monthly interval for 9 months (36 weeks). A total of 36 samples were collected at each time point on three selected plants for each variety-virus combination (i.e., 3 plants × 2 samples per plant × 3 varieties × 2 viruses = 36). This resulted in the collection of a total of 144 (36 samples × 4 collection times) samples in the first week after graft-inoculation, followed by weekly collections for three weeks 108 (36 weekly samples x 3 weeks) by the end of 4 weeks. After four weeks 288 samples were collected at four weeks intervals (36 samples × 8). Overall a total of 540 roots and leaf samples were collected by the end of 36 weeks (9 months) and 468 were analysed for virus detection, movement and concentrations. Samples were tested by RT-PCR.

### 6.2.4 RT-qPCR

Total nucleic acids were extracted from cassava samples as described previously in Chapter 3 (section 3.2.7). The quality and quantities of RNA in each sample was assessed using a Biophotometer (eppendorf, UK). ImProm-II$^{TM}$ Reverse

Transcriptase kit was used following the manufacturer's instructions (Promega, UK) for cDNA synthesis. The amount of RNA used in each cDNA synthesis reaction was 1 µg as recommended by Moreno *et al*. (2011). Samples were DNase-treated using RNase-free DNase RQ 1 treatment kit (Promega, USA) according to manufacturer's instructions to remove DNA. The DNase treated samples were used for first strand cDNA synthesis for real-time reverse transcription-quantitative PCR (RT-qPCR) (Bustin *et al*., 2009). To minimize any errors due to pipetting differences, cDNA was synthesised in duplicates of each sample and their threshold cycle (Ct) values were averaged during data analysis as described by Kokkinos and Clark (2006). In addition, every plate included a non-template water control (NTC). Three µl of random primer mix (New England Biolabs, UK) was used in the first master mix. The cDNA synthesis protocol for the second master mix was the same as described (section 3.2.8).

### 6.2.5 Measuring virus titres in cassava

For quantification of the virus titre in cassava varieties; the RT-qPCR method described by Moreno *et al*. (2011) was used to quantify gene expression in Albert, Kiroba and Kaleso. CBSVs-specific primers, forward (Abarshi *et al.*, 2012) and reverse were used for virus amplification (Table 6.1). Previously identified reference genes, ribulose biphosphate carboxylase oxygenase gene (RubiscoL) and the ribosomal protein (L2) were used as internal controls for data normalization. Primers used were RubiscoLF and RubiscoLR designed to amplify a PCR product size of 171 bp (Nassuth *et al*., 2000; Alabi *et al*., 2008; Abarshi *et al*., 2012) and L2F and L2R with PCR fragment of 135 bp (Nicot *et al*., 2005). A typical qPCR reaction mixture contained a total volume of 25 µl (Table 6.2). The mixture was dispensed into qPCR plates using robot Ep Motion 5070 (Hamburg, Germany) to avoid pipeting error. The qPCR plates were sealed using adhesive Master clear qPCR film (eppendorf, UK) to provide protection against evaporation. Thermal cycling conditions used in qPCR are described below (Table 6.3): The qPCR reactions were performed with the Master Cycler Ep RealPlex PCR system (Hamburg, Germany) using the SDS software for data measurement and analysis.

Table 6.1: Primers used in qPCR reactions for the quantification of UCBSV and CBSV in cassava varieties

| Primer name | Primer sequence (5´-3´) | Product size | Reference |
|---|---|---|---|
| CBSVF3 | GGARCCRATGTAYAAATTTGC | 130 bp | Abarshi *et al*., 2012 |
| CBSVR4[a] | GCWGCTTTTATYACAAAMGC | | |
| RubiscoLF | CTTTCCAAGGCCCGCCTCA | 171 bp | Nassuth *et al*., 2000 |
| RubiscoLR | CATCATCTTTGGTAAAATCAAGTCCA | | Alabi *et al*., 2008 |
| L2F | TGGTGTTGCCATGAACCCTGTAGA | 135 bp | Nicot *et al*., 2005 |
| L2R | CGACCAGTCCTCCTTGCAGC | | |

Table 6.2: Reaction mixture for qPCR quantification of the viral cDNA

| Reagent | × 1 sample (µl) |
|---|---|
| SDW | 8.5 |
| SYBR Green (1000×) | 12.5 |
| Forward primers (5 µM) | 1.5 |
| Reverse primers (5 µM) | 1.5 |
| cDNA template | 2.5 |
| Total | 25.0 |

Table 6.3: Temperature profile and thermal cycling conditions

| Steps | Temperature ($^{o}$C) | Time | Number of cycles |
|---|---|---|---|
| Initial denaturetion | 95 | 15 min | × 40 cycles |
| Final denaturation | 94 | ¼ min | |
| Annealing | 55 | ½ min | |
| Extension | 72 | ½ min | |

### 6.2.6 Data analysis from RT-qPCR

For data analysis the default settings of the Master Cycler Ep Realplex PCR system software were used and qPCR efficiency was calculated based on the raw fluorescence data (ΔRn) exported as output file and subsequently imported into the qPCR program (Ruijter *et al*., 2009). Relative quantifications were performed based on the cycle threshold (Ct) method described by Livak and Schmittgen (2001). The Ct value is defined as the cycle number at which the ΔRn crosses the threshold. The fold change in virus (target gene) relative to the reference gene (RubiscoL) was determined by the Ct formula given as:

$Ct = 2^{\wedge\text{-}\Delta\Delta Ct}$: $Ct = 2^{-}[(Ct_{\text{target gene}}) - (Ct_{\text{reference gene}})] - [(\text{mean } Ct_{\text{target gene}}) - (\text{mean } Ct_{\text{reference gene}})]$

Where: Ct = threshold cycle, ΔΔCt = Mean fold change. The geometric averaging of one of the two internal controls was used for data normalization. Leaf samples collected from the three cassava plants in each variety were pooled according to the protocol described by Nicot *et al.* (2005) and used as templates in the qPCR assay and their Ct values were compared. Adequate performance of the qPCR method was confirmed by low standard deviations for technical duplicates. A comparison between qPCR normalized data for each infected variety was done using the Microsoft Excel and the data presented to indicate relative virus load in each variety at each time point. The shifted Gompertz model was tested to determine its appropriateness to describe the virus titre progress curve (Appendix 1.3) from the same Ct data using the formula:

$$Ct = bze^{-\eta z}(1+\eta\,(1-z))$$

Where $z = e^{-bt}$, b = the scale parameter, η = the shape parameter, t = time (in weeks) and a constant multiplier (e = 2.7183).

### 6.2.7 Assessment of reversion in CBSD-infected cassava varieties

Stem cuttings from Kaleso, Kiroba and Albert were obtained from 20 months old CBSD-infected plants. For each virus-variety combination, 54 cuttings of 10 cm were made from graft-inoculated plants of each variety (section 6.2.2) and planted into plastic pots containing John Innes No. 2 compost. The plants were grown in the quarantine glasshouse. The proportion of the plants that sprouted

from each variety per isolate was recorded monthly. The term reversion is used to describe the production of symptom-free plants from cuttings derived from diseased plants (Fondong *et al*., 2000). The rate of reversion in CBSD-infected cuttings was assessed based on the proportion of plants that did not develop symptoms upon sprouting and grown up to six months after planting. Symptoms were recorded weekly by visual observations. The presence or absence of UCBSV and CBSV on symptom-free plants (by visual observation) in each variety was further confirmed by RT-PCR at six months after planting. The role of several parameters including the length of the cassava stem cutting and the position of the stem (upper, middle and smaller) were investigated;

**Length of stem cuttings on reversion**: Different lengths of stem cuttings were taken from CBSD-infected plants of Kaleso, Kiroba and Albert. Cuttings were made short (10 cm), intermediate (15 cm) and long (20 cm) pieces and planted in pots of 0.5 litres. Twenty cuttings were made for each length-variety-virus combinations and were replicated thrice. The rate of reversion was assessed as the percentage of disease-free plants obtained six months after planting.

**Effect of cutting position on reversion**: Stems were taken from CBSD-affected plants and divided into; lower (woody stem), middle (rigid stem) and upper (soft stem) parts. From each part of the plant, 10 cm cuttings were made and grown in the NRI quarantine glasshouse (Figure 6.1). Eighteen cuttings were planted for each stem position and virus-variety combinations and the experiment was repeated three times using the same mother plant as source material. Plants were observed for symptoms for up to six months. Reversion in different stem positions was compared using a multiple comparison (ANOVA) to determine the effects of stem position on reversion.


**Figure 6.1**: Cassava stems planted as part of CBSD reversion experiment.

### 6.2.8 Whitefly fecundity studies to determine the mechanisms of resistance

The colony of cassava *B. tabaci* used in this study was originally obtained from cassava in Uganda (Maruthi *et al*., 2001, 2002). A total of 1000 *B. tabaci* adults were collected from the cages and released into a new cage of 112 x 50 x 50 cm, containing two young cassava plants of the variety Columbian. *B. tabaci* were reared in the new cage for four weeks to obtain adequate number needed for the experiment (Figure 6.2). Adult whiteflies of mixed sex likely (5:5 male to female) were collected and transferred into a clip cage which was attached to the leaf of a cassava plant. The top five leaves of 10 plants were inoculated for each variety Albert, Kiroba and Kaleso. Fifty treatments were made for each variety in a three replicate experiment to give a total of 450 inoculated leaves (10 plants x 5 leaves x 3 varieties x 3 replications). Whiteflies were allowed to lay eggs for 48 h and then removed mechanically. Plants were kept in the insectary at 28 ± 2 $^{o}$C and 60% RH and L12:D12 for the egg to develop into adults. The fecundity and survival of *B. tabaci* was recorded by measuring the number of eggs laid, the nymphs developed and the adults emerged on each cassava variety by counting under a stereo binocular microscope. The data obtained were analysed by ANOVA to determine the effect of varieties on the fecundity of *B. tabaci.* The percentage response of eggs that survived to nymphs, nymphs to adults and eggs to adults was calculated from the logit estimates using the formula:

$Pr = \{(e^{x}) / (1+ e^{x})\} \times 100$

Where x = logit, Pr = percentage response and the constant e = 2.7183.

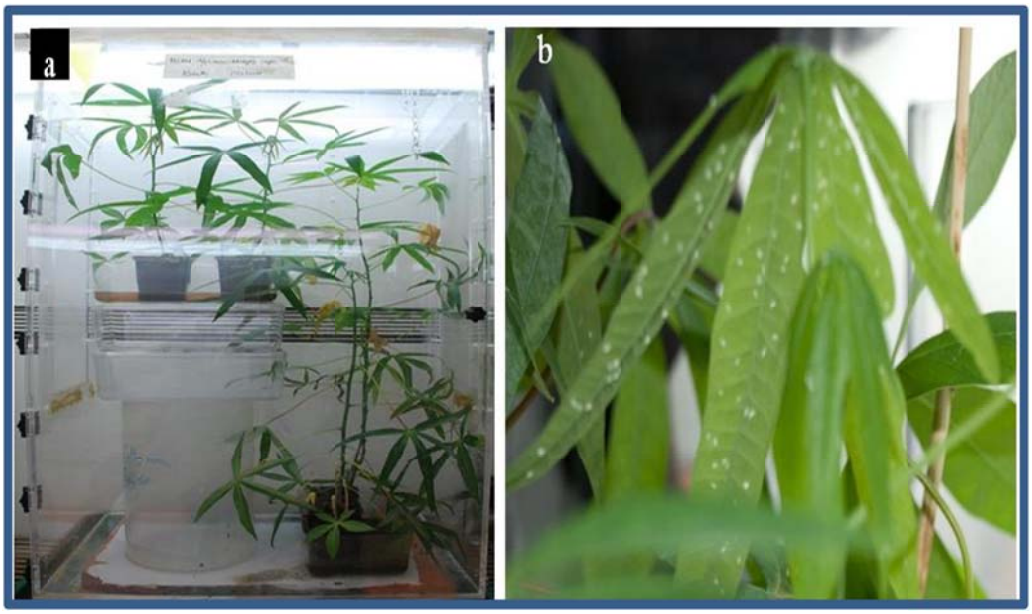



**Figure 6.2**: (a) *B. tabaci* colony maintained in the NRI insectary, (b) *B. tabaci* adults (white spots) on the newly opened cassava leaf in the colony.

### 6.2.9 Whitefly inoculation studies

This experiment was carried out to determine the susceptibility of the three cassava varieties when CBSV was transmitted by whiteflies. Approximately 1000 adult *B. tabaci* were collected from the colony and introduced into a cage (dimension 60 cm long x 60 cm wide x 90 cm high) for 24 h containing three young cassava plants of var. Ebwanateraka infected with CBSV-[MZ:Nam1-1:07] (Figure 6.3). *B. tabaci* adults were then transferred into the clip-cages placed onto leaf number three of the test plants, for 24h of virus inoculation access period (IAP, Approximately 30).

Thirty treatments were made per variety in a three replicate experiment to give a total of 90 inoculated plants (10 plants x 3 varieties x 3 replications). The *B. tabaci* were removed mechanically after inoculation. These were maintained for six months at 28 ± 2 $^{o}$C and 60% RH. CBSD symptom development was monitored on inoculated leaves and on entire recipient plants. The efficiency of transmission was determined as proportion of infected plants expressed as a percentage of the total number of plants tested.

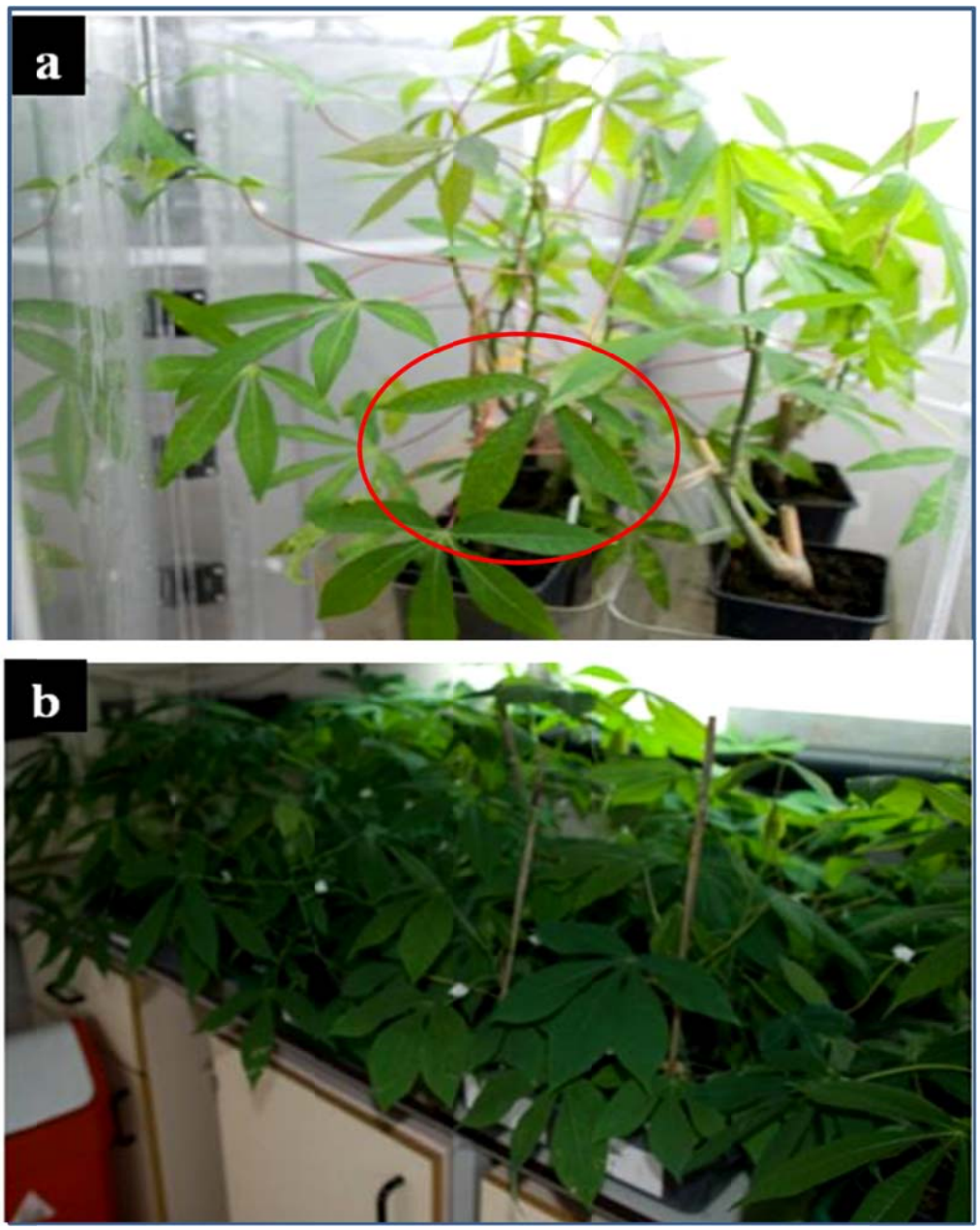



**Figure 6.3:** (a) CBSD-infected plants of var. Ebwanateraka used as virus source on which whiteflies were allowed to feed freely on symptomatic leaves (example leaf circled red) in the cage, and (b) CBSV-transmitted plants incubated for symptom expression.

## 6.3. Results

### 6.3.1 Rate of graft-transmission, symptoms development and severity on cassava varieties

Plants graft-inoculated with the CBSV-[MZ:Nam1-1:07] became infected in less than two weeks and the rate of transmission varied among cassava varieties (Table 6.4). Development of symptoms on graft-inoculated plants varied between the isolates. CBSV-[MZ:Nam1-1:07] produced the earliest and greatest rate of transmission on all three cassava varieties (Table 6.4). All inoculated plants were infected at 16 weeks after graft-inoculation.

CBSD symptoms on cassava leaves were observed on all the three varieties. The type of leaf symptoms expressed by each cassava variety depended on the isolate and they were similar to the ones observed in previous experiments, in which relatively milder symptoms were expressed by the plants infected with UCBSV-[UG:Kab4-3:07] compared to the severe symptoms by CBSV-[MZ:Nam1-1:07].

Both virus isolates produced lesions on stems on all plants of Albert, and only 40% of the Kiroba plants produced CBSV-[MZ:Nam1-1:07] symptoms (Figure 6.4a and b). Stem symptoms were seen on Albert but not on Kaleso by either isolate (Figure 6.5a and b).

Depending on the isolate, root necrosis was observed on all the cassava varieties, albeit with differing severity. Root necrosis was found in all the roots harvested from Albert with the two viruses. Roots harvested from Kaleso infected with UCBSV-[UG:Kab4-3:07] were symptomless while CBSV-[MZ:Nam1-1:07] infections did cause small necrotic dots (Figure 6.6, 6.7 and 6.8). The scores for the root symptom severity were on average 1.8 for UCBSV-[UG:Kab4-3:07] and 2.3 for CBSV-[MZ:Nam1-1:07]. The lowest score was recorded on Kaleso while the greatest on Albert (scores 3 and 4). The mean root severity score on Kiroba was 1.5 for CBSV-[MZ:Nam1-1:07] and 1.7 for UCBSV-[UG:Kab4-3:07] (Figure 6.9).

Table 6.4: Rate of symptoms development of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] on cassava varieties.

| Cassava varieties | Number of grafts[a] | Number grafted[b] | | Number infected/Number re-grafted | | Proportion (%) | |
|---|---|---|---|---|---|---|---|
| | | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Kaleso | 1st | 5 | 5 | 0/5 | 0/5 | 0 | 0 |
| | 2nd | 5 | 5 | 0/5 | 3/5 | 0 | 60 |
| | 3rd | 5 | 2 | 2/5 | 2/2 | 40 | 100 |
| | 4th | 3 | - | 3/3 | - | 100 | - |
| Kiroba | 1st | 5 | 5 | 0/5 | 2/5 | 0 | 40 |
| | 2nd | 5 | 3 | 2/5 | 3/3 | 40 | 100 |
| | 3rd | 3 | - | 3/3 | - | 100 | - |
| | 4th | - | - | - | - | - | - |
| Albert | 1st | 5 | 5 | 4/5 | 5/5 | 80 | 100 |
| | 2nd | 1 | - | 1/1 | - | 100 | - |
| | 3rd | - | - | - | - | - | - |
| | 4th | - | - | - | - | - | - |

[a]Number of repeated graftings on cassava varieties.
[b]Number of plants grafted for each repeated grafting in each variety and isolate
- indicated no grafting was done on the variety because all the plants in that variety expressed CBSD with previous grafting.

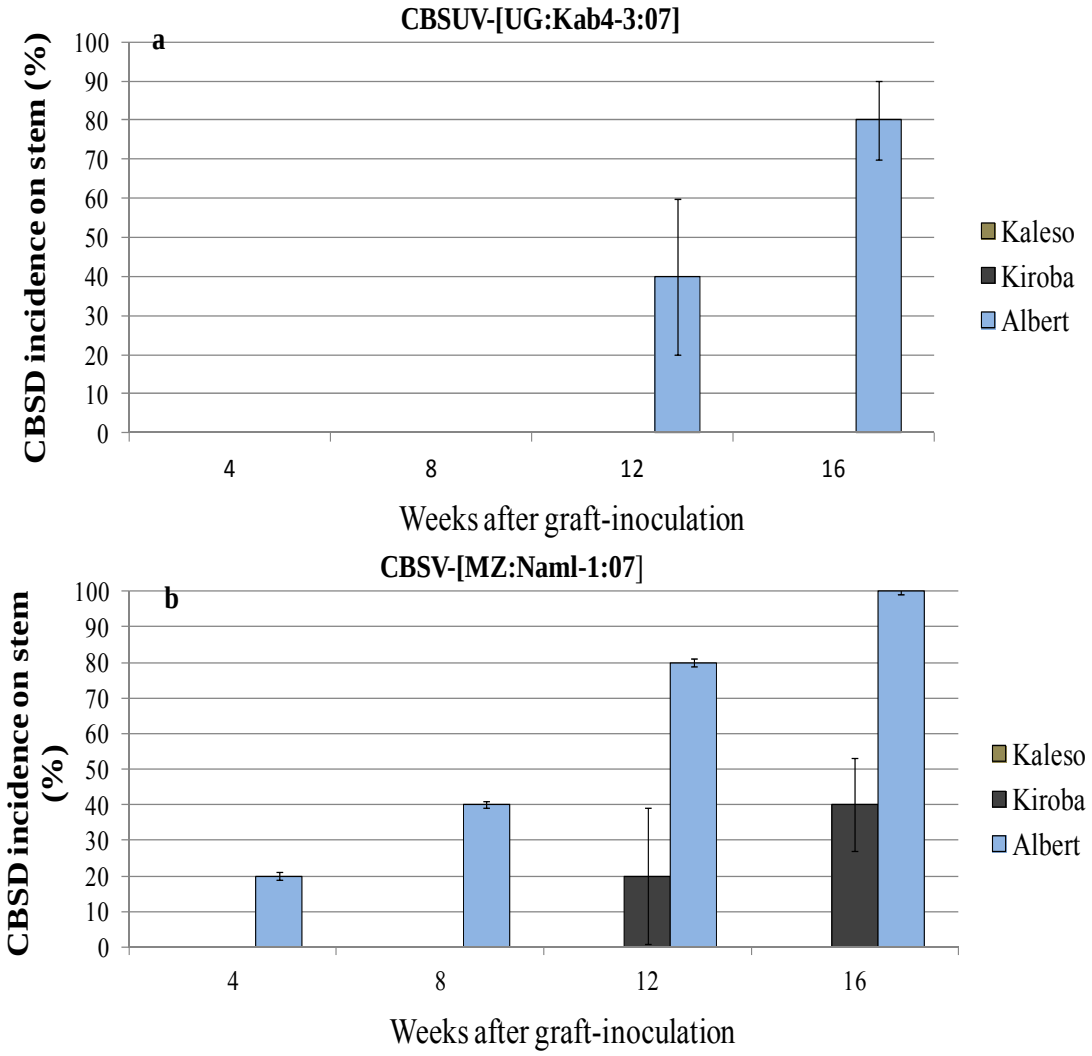



**Figure 6.4:** CBSD symptoms on stems of three cassava varieties.

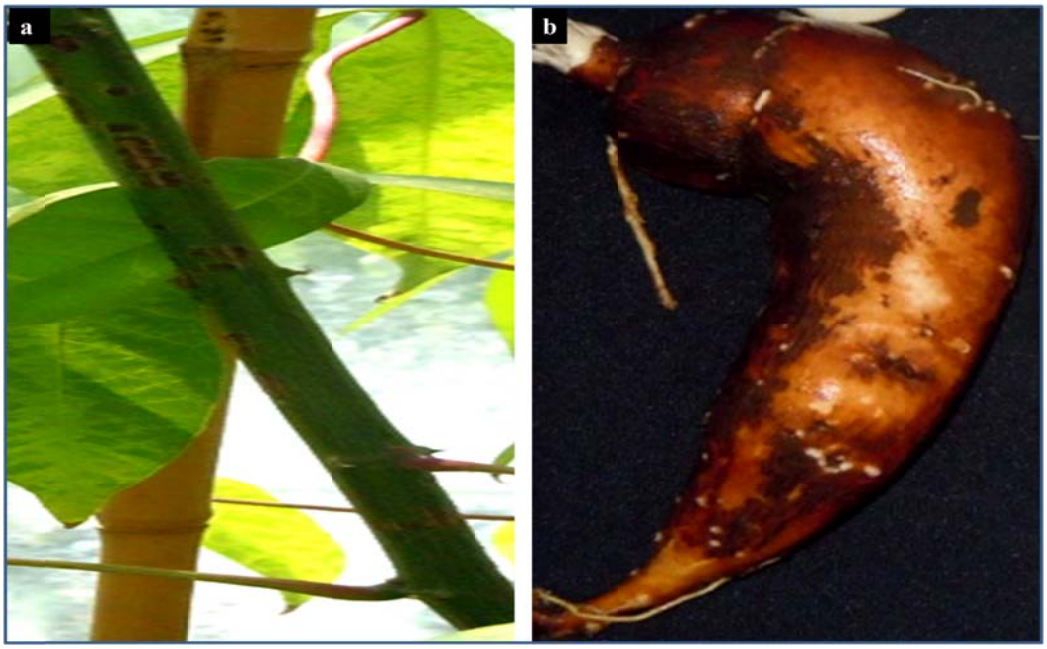



**Figure 6.5**: (a) CBSD stem symptoms observed on Albert, (b) example of the harvested root of Kaleso 2.5 years after planting.

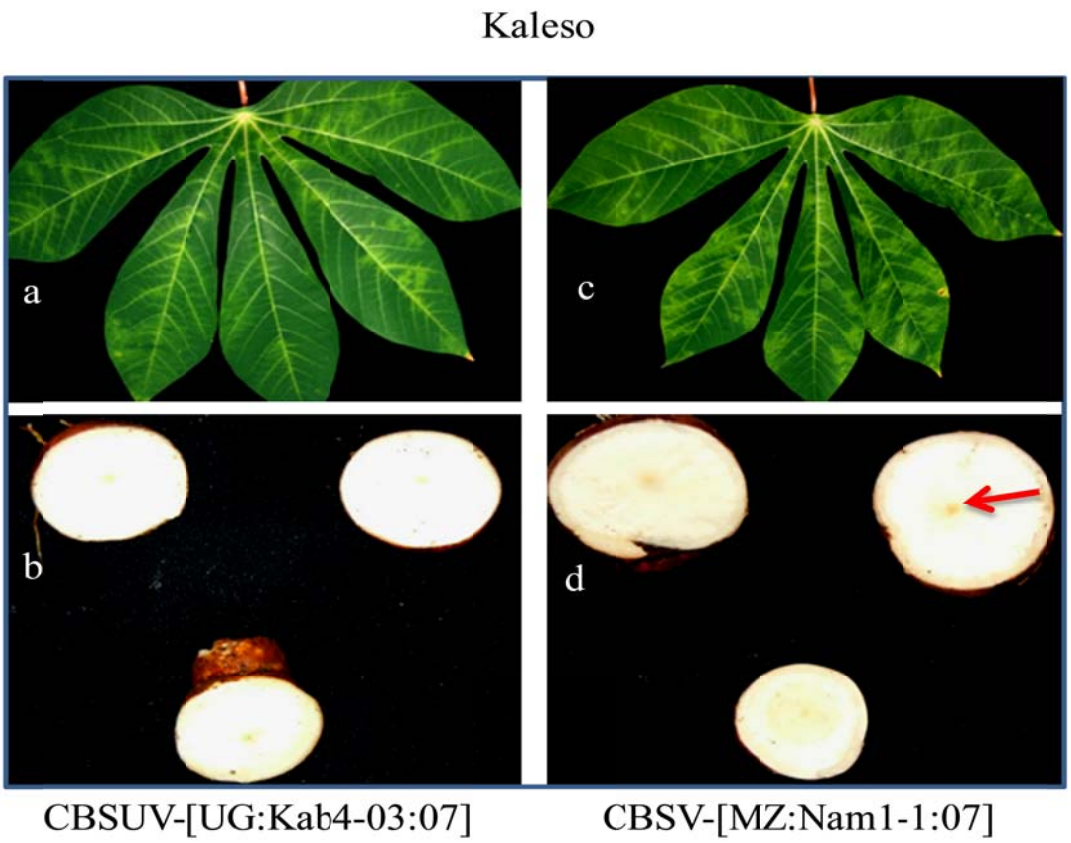



**Figure 6.6**: Leaf and root symptoms by UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] on Kaleso with mild necrosis (red arrow).

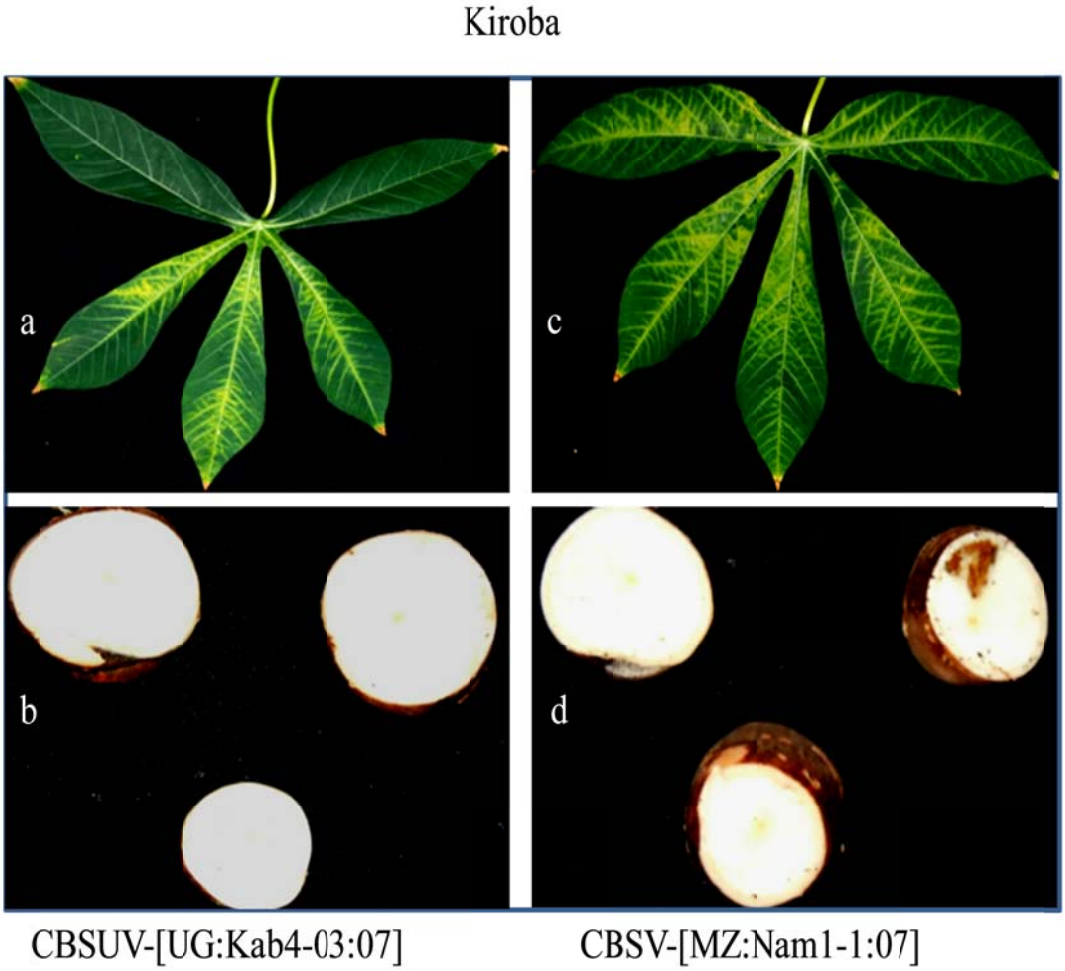



**Figure 6.7:** Leaf and root symptoms by UCBSV-[UG:Kab4-3:07] (a and b) and CBSV-[MZ:Nam1-1:07] (c and d) on Kiroba.

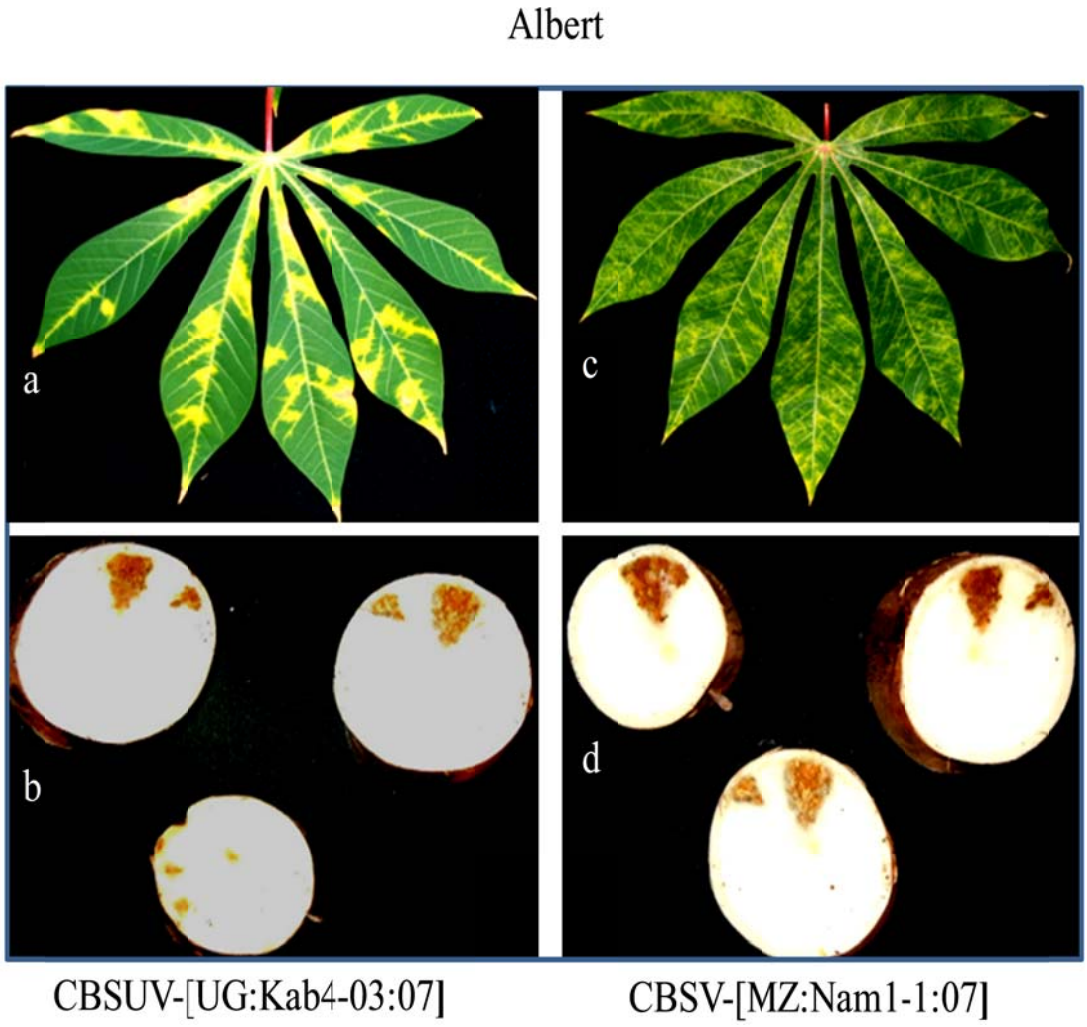



**Figure 6.8**: Leaf and root symptoms on Albert by UCBSV-[UG:Kab4-3:07] (a and b), and CBSV-[MZ:Nam1-1:07] (c and d).

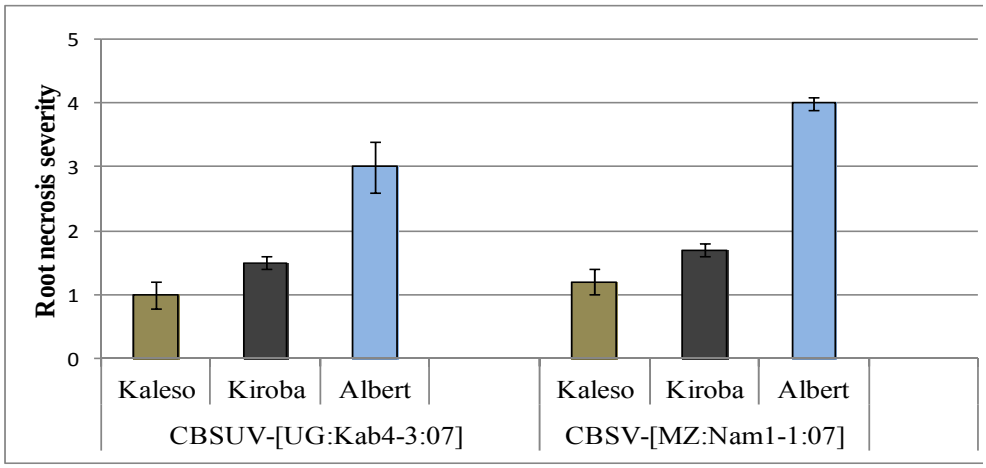



**Figure 6.9**: Symptom severity recorded on the roots of three cassava varieties for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. Symptom severity was based on a scale of 1 (no symptoms) to 5 (very severe symptoms) (Hillocks *et al*., 2001; McSween *et al.,* 2006).

### 6.3.2 Virus detection and movement within cassava varieties

UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] were not detected from the leaves in any of the plants 24 and 48 h after graft-inoculation. CBSV-[MZ:Nam1-1:07] was first detected in the roots from 1 out of 3 plants in Albert at four days after graft-inoculation, which indicated that the first movement of the virus from the graft-inoculation point was down to the roots (Figure 6.10a and b). None of the samples from Kaleso and Kiroba inoculated with CBSV-[MZ:Nam1-1:07] tested positive by RT-PCR at four days after graft-inoculation. Similarly, UCBSV-[UG:Kab4-3:07] was not detected in any of the samples at four days after graft-inoculation (Figure 6.10a and b).

Both viruses were detected at one week after graft-inoculation from both leaves and roots in Albert, while at 12 weeks both viruses were detected in roots and leaves of all the sampled plants in all the three varieties (Figure 6.11a, b, c and d). At 28 weeks, only two of the three Kaleso plants had UCBSV-[UG:Kab4-3:07] in the roots, while the virus was fluctuating in leaves. Like in Kaleso, the number of roots that had virus varied at 36 weeks in Kiroba (Figure 6.12c; Table 6.5). Albert did not show the fluctuation in the number of samples containing the viruses up to 36 weeks after graft-inoculation (Table 6.5).

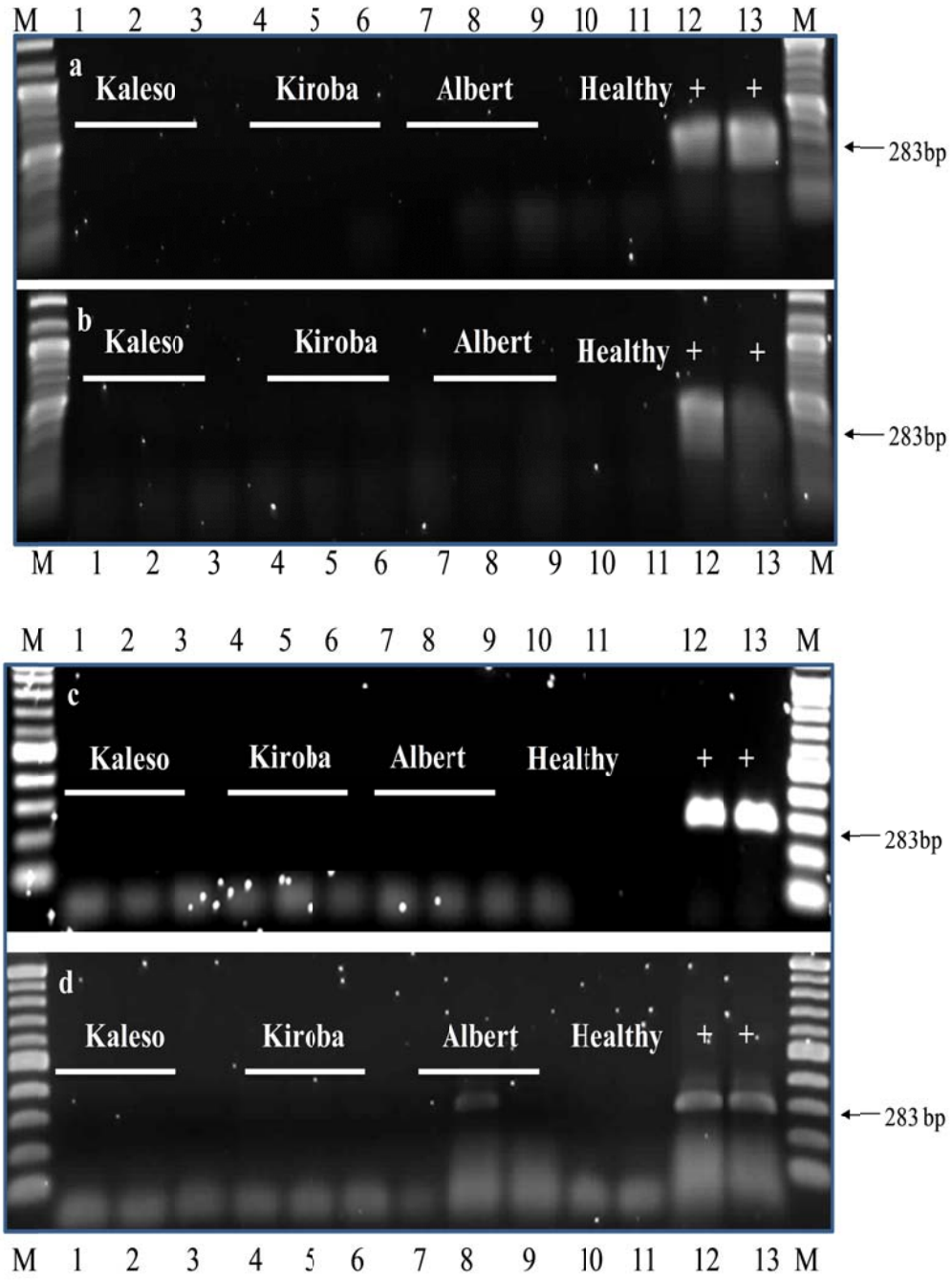



**Figure 6.10**: Detection of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] at four days after graft-inoculation in top leaves (a and b), and roots (c and d) of three cassava varieties using RT-PCR using CBSVF3 and CBSVR3 primers. RNA isolated from graft-inoculated plants in (a) UCBSV-[UG:Kab4-3:07], (b) CBSV-[MZ:Nam1-1:07] (from top leaves), (c) UCBSV-[UG:Kab4-3:07] and (d) CBSV-[MZ:Nam1-1:07] (from the roots). Lanes 1, 2 and 3 = Kaleso, 4, 5 and 6 = Kiroba, 7, 8 and 9 = Albert, 10 and 11 = healthy cassava plants used as control, 12 and 13 = known CBSV RNA controls. The size ladder (M) at each border of the gels is the 100 bp molecular weight markers (New England Biolabs, UK). CBSV-[MZ:Nam1-1:07] was detected at four days after graft-inoculation in 1 of 3 samples from Albert lane 8.

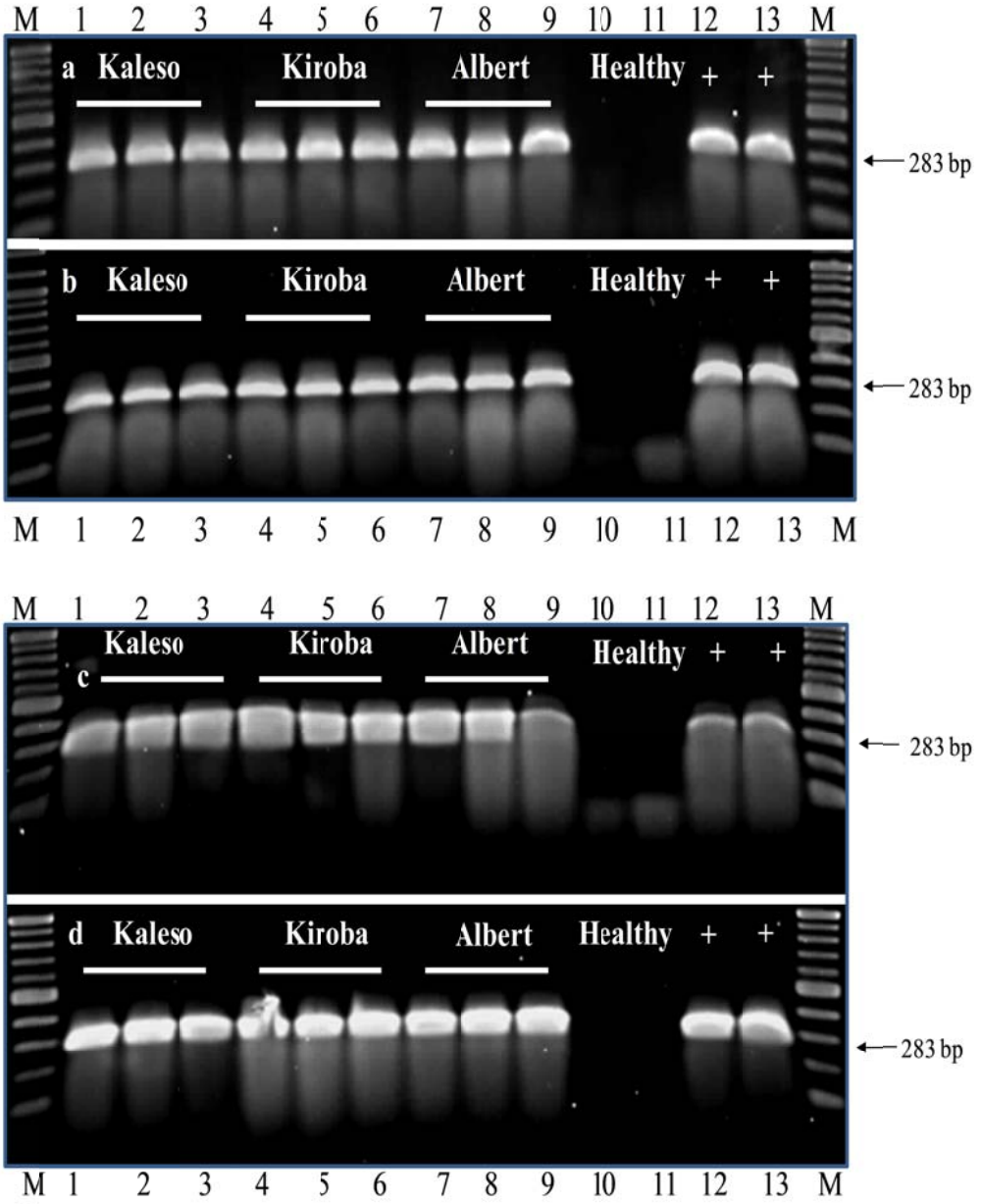



**Figure 6.11**: Detection of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] at 12 weeks after graft-inoculation in top leaves (a and b) and roots (c and d) of three cassava varieties by RT-PCR using CBSV F3 and CBSV R3 primers. RNA isolated from graft-inoculated plants in (a) UCBSV-[UG:Kab4-3:07], (b) CBSV-[MZ:Nam1-1:07] (from top leaves), (c) UCBSV-[UG:Kab4-3:07] and (d) CBSV-[MZ:Nam1-1:07] (from the roots). Lanes 1, 2 and 3 = Kaleso, 4, 5 and 6 = Kiroba, 7, 8 and 9 = Albert, 10 and 11 = healthy cassava plants used as control, 12 and 13 = known CBSV RNA controls. The size ladder (M) at each border of the gels is the 100 bp molecular weight markers (New England Biolabs, UK).

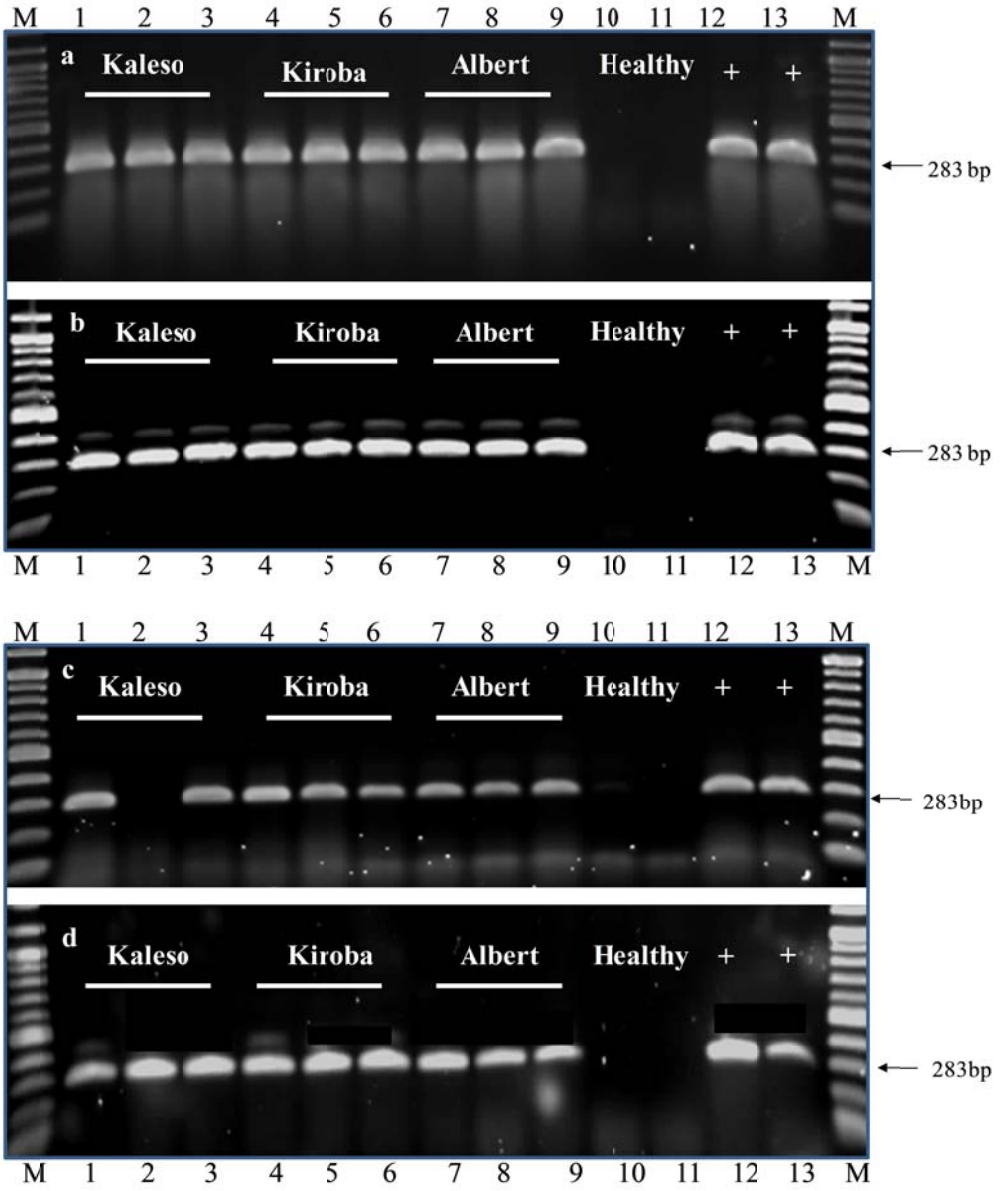



**Figure 6.12**: Detection of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] at 36 weeks after graft-inoculation in (a and b) top leaves (c and d) roots of three cassava varieties using the two-step RT-PCR using CBSV-specific primer pair CBSV-F3 and CBSV-R3. RNA isolated from graft-inoculated plants in (a) UCBSV-[UG:Kab4-3:07], (b) CBSV-[MZ:Nam1-1:07] (from top leaves), (c) UCBSV-[UG:Kab4-3:07] and (d) CBSV-[MZ:Nam1-1:07] (from the roots). Lanes 1, 2 and 3 = Kaleso, 4, 5 and 6 = Kiroba, 7, 8 and 9 = Albert, 10 and 11 = healthy cassava plants used as control, 12 and 13 = known CBSV RNA controls. The size ladder (M) at each border of the gels is the 100 bp molecular weight markers (New England Biolabs, UK).

Table 6.5: Movement of CBSVs from the point of inoculation to other parts of the plants

| | Number of plants positive by RT-PCR/Number tested[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | | | | CBSV-[MZ:Nam1-1:07] | | | | | |
| | Kaleso | | Kiroba | | Albert | | Kaleso | | Kiroba | | Albert | |
| Weeks | leaf | root | leaf | root | leaf | root | leaf | root | leaf | root | leaf | root |
| 1 | 0/3 | 0/3 | 0/3 | 2/3 | 1/3 | 1/3 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 2/3 |
| 2 | 0/3 | 1/3 | 0/3 | 2/3 | 0/3 | 2/3 | 0/3 | 0/3 | 1/3 | 2/3 | 1/3 | 2/3 |
| 4 | 0/3 | 0/3 | 0/3 | 0/3 | 1/3 | 3/3 | 0/3 | 0/3 | 1/3 | 2/3 | 3/3 | 3/3 |
| 8 | 2/3 | 2/3 | 3/3 | 2/3 | 3/3 | 3/3 | 2/3 | 1/3 | 1/3 | 2/3 | 3/3 | 3/3 |
| 12 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 3/3 | 3/3 |
| 16 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 20 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 24 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 28 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 32 | 2/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| 36 | 3/3 | 2/3 | 3/3 | 2/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 |

[a]Number of plants infected by UCBSV-[UG:Kab4-3:07] and for CBSV-[MZ:Nam1-1:07] after graft-inoculation in the three cassava varieties, Kaleso, Kiroba, and Albert.

### 6.3.3 Measuring virus titres in three cassava varieties

Titres of CBSV-[MZ:Nam1-1:07] were greater in all the three cassava varieties than UCBSV-[UG:Kab4-3:07] (Figures 6.13 and 6.14). Among cassava varieties, Albert showed the greatest virus titre compared to Kiroba and Kaleso, which showed medium and low level of titres, respectively. Virus titres did not vary considerably throughout the sampling periods in Kaleso, while UCBSV-[UG:Kab4-3:07] increased from 1 at one week to about 13.7 fold at 16 weeks after graft-inoculation in Kiroba (Figure 6.13). The expression of CBSV-[MZ:Nam1-1:07] in Kiroba also followed similar trend, which ranged from 1 fold at one week to 41.9 fold at 24 weeks after grafting. In CBSV-[MZ:Nam1-1:07]-infected plants of Albert, the virus titre increase consistently from 1 fold at week one to about 281.7 fold at 36 weeks after graft-inoculation, which is consistent with severe symptoms (section 6.3.1).

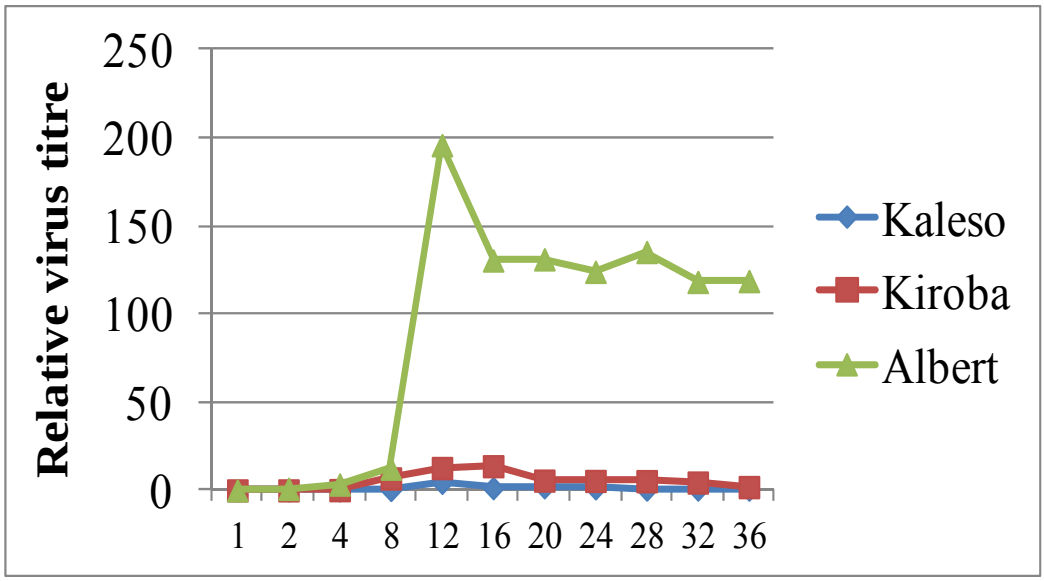



**Figure 6.13**: Mean fold change in UCBSV-[UG:Kab4-3:07] titres (Change in expression level = 2^-ΔΔCt) over time (weeks) in Kaleso, Kiroba and Albert.

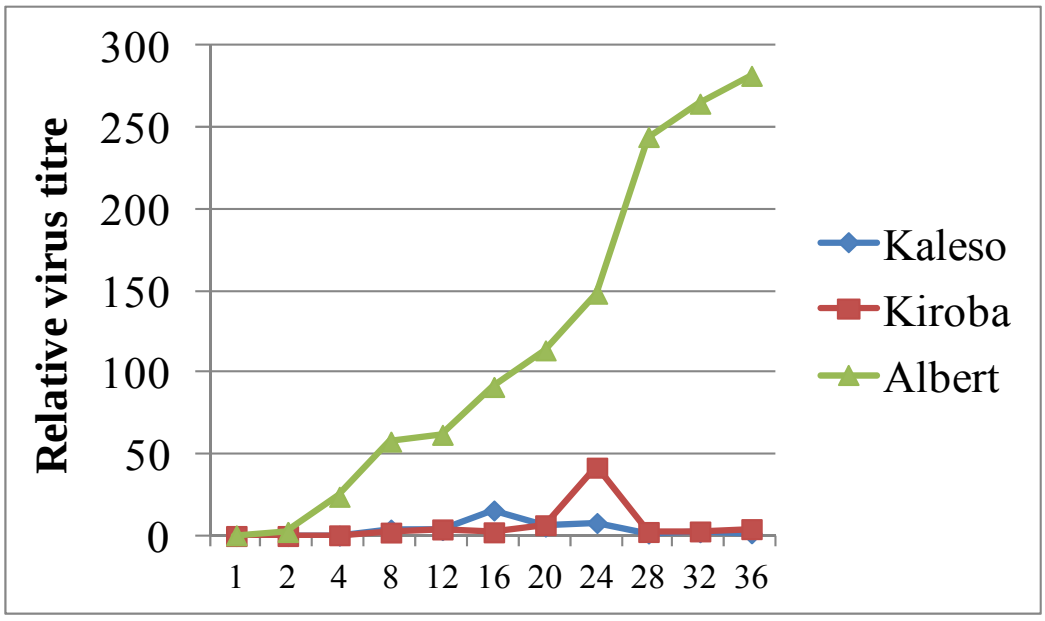



**Figure 6.14**: Mean fold change in CBSV-[MZ:Nam1-1:07] titres (Change in expression level = 2^-ΔΔCt) over time (weeks) in Kaleso, Kiroba and Albert.

### 6.3.4 Assessment of reversion on CBSD-infected cuttings

About 80% of UCBSV-[UG:Kab4-3:07]- and 75% of CBSV-[MZ:Nam1-1:07]-infected cuttings sprouted and grew fully from Kaleso. From Kiroba (75 and 64%) and Albert (69 and 57%), a decreasing number of cuttings sprouted (Figure 6.15), while the remaining cuttings died. Reversion was observed on all the three cassava varieties, which was confirmed at six months after planting, when all the cassava plants not showing CBSD symptoms were tested by RT-PCR and found negative for the virus. Similarly, significant differences ($P<0.0004$) in the reversion were observed between the three cassava varieties. In Albert, a smaller percentage of reversion was recorded in UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-infected cuttings (18 and 12% respectively). This was followed by Kiroba (21 and 16%). Kaleso (38 and 23%) showed significantly high percentage of plants ($P<0.001$) recovered from UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-infections (Figures 6.16 and 6.17).

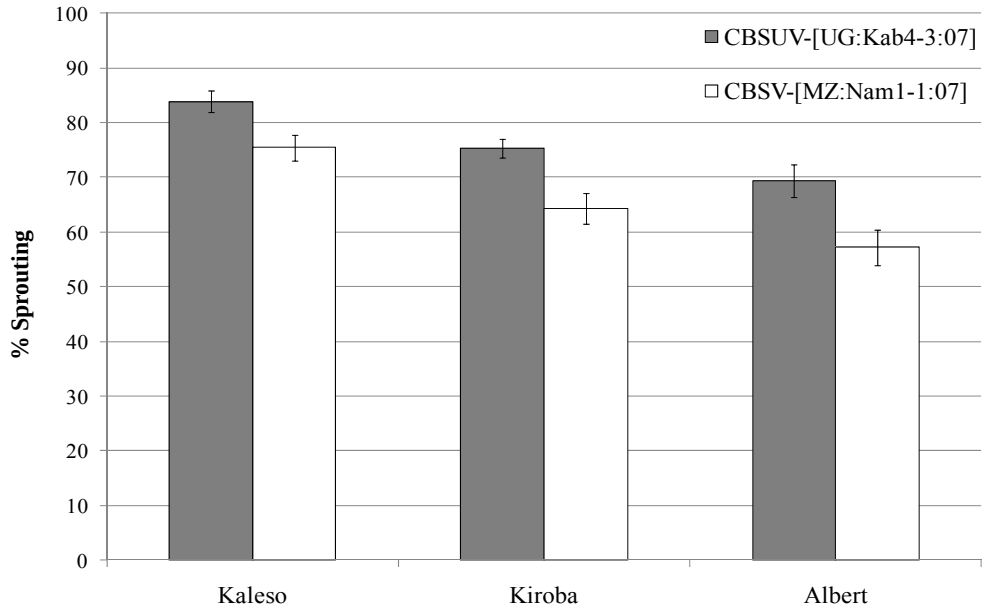



**Figure 6.15**: Proportion of cuttings sprouted from virus-affected cassava varieties. n = 54 for each variety.

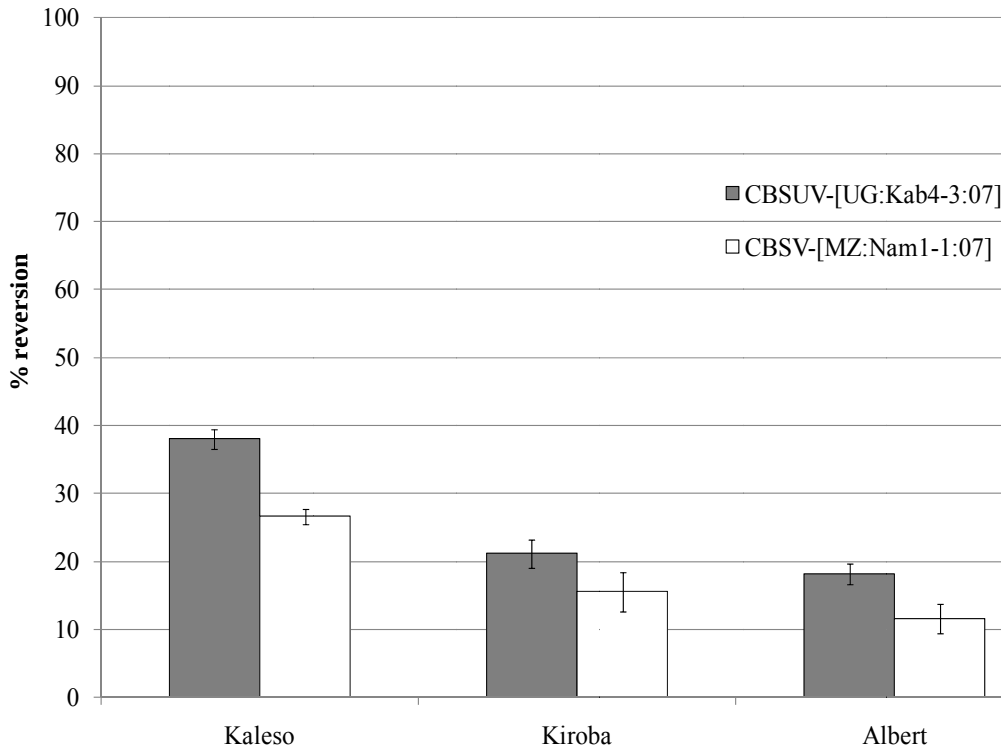



**Figure 6.16:** Proportion of disease-free plants that grew from virus-affected cuttings as an effect of reversion, n = 54 cuttings for each variety.

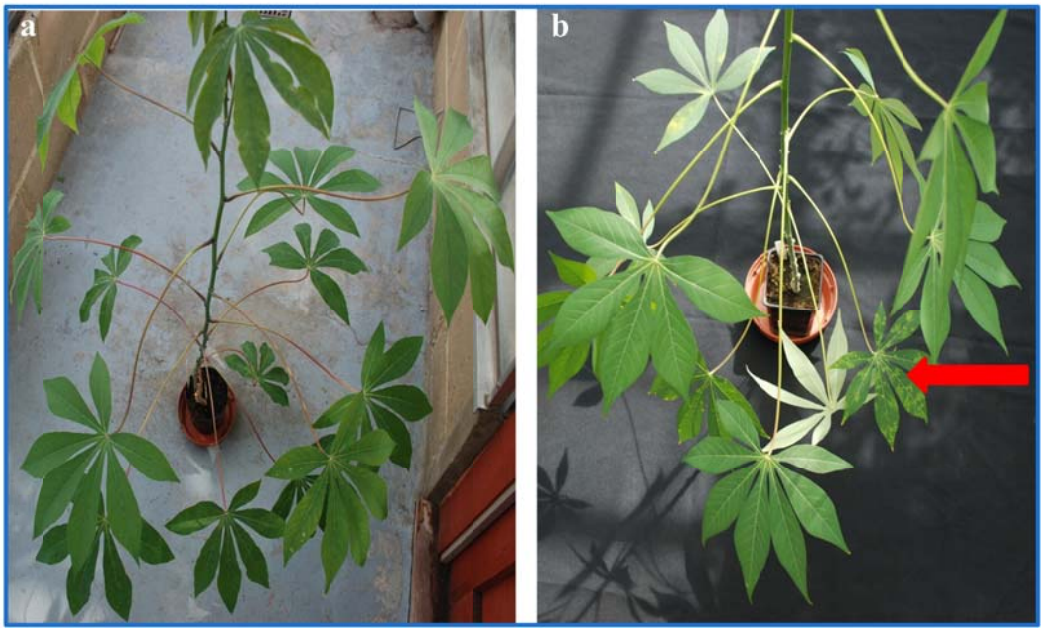



**Figure 6.17:** Plants of varieties Kaleso (a) showing reversion (loss of symptoms) from CBSD and Albert (b) has not lost the virus five months after planting in the NRI quarantine glasshouse.

### 6.3.5 Effect of stem cuttings on plant regeneration and rate of reversion

About 68% of cuttings (from 10 cm length) infected with UCBSV-[UG:Kab4-3:07] each for vars. Kaleso and Kiroba, were sprouted and grown fully into cassava plants. The percentage of cuttings that were sprouted in Albert was 65%. The growth of plants from UCBSV-[UG:Kab4-3:07]-infected cuttings of 15 cm length were 90%, 92% and 80% from Kaleso, Kiroba and Albert respectively. The percentage of cuttings that sprouted from UCBSV-[UG:Kab4-3:07]-infected cuttings of 20 cm were greater than in 10 cm and 15 cm (Table 6.6).

The sprouting of plants from CBSV-[MZ:Nam1-1:07]-infected cuttings also followed similar pattern with greater number of cuttings been grown from 20 cm long cuttings than 10 cm and 15 cm (Table 6.7). There was considerable variation in the reversion resulting from stem cuttings of different lengths. Ten cm cuttings resulted in most reversion, followed by cuttings measuring 15 cm and reversion was least in 20 cm stem cuttings. Stem cuttings from plants of UCBSV-[UG:Kab4-3:07] infection gave more reversion compared to those from CBSV-[MZ:Nam1-1:07].

Table 6.6: Effect of CBSD on the sprouting of cassava cuttings of different length.

| % plants sprouted from each cutting length | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | | CBSV-[MZ:Nam1-1:07] | | | |
| | Cuttings length | | | | Cuttings length | | | |
| | 10 cm | 15 cm | 20 cm | Mean[a] | 10 cm | 15 cm | 20 cm | Mean[a] |
| Variety | % | % | % | % | % | % | % | % |
| Kaleso | 68 | 90 | 97 | 85 | 67 | 88 | 92 | 82 |
| Kiroba | 68 | 92 | 95 | 85 | 62 | 90 | 85 | 78 |
| Albert | 65 | 80 | 95 | 80 | 57 | 72 | 85 | 72 |
| Mean[b] | 67 | 87 | 95 | | 62 | 83 | 87 | |

[a]Mean of cuttings that were sprouted across different cuttings length.
[b]Mean of cuttings that were sprouted across three varieties for different cuttings sizes.

Table 6.7: Assessment of reversion of CBSD-infected cuttings of different length

| | % virus-free plants at six months after planting[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | RT-PCR | CBSV-[MZ:Nam1-1:07] | | | RT-PCR |
| | Cuttings length | | | Mean[b] | Cuttings length | | | Mean[b] |
| Variety | 10 cm | 15 cm | 20 cm | % | 10 cm | 15 cm | 20 cm | % |
| Kaleso | 42 | 37 | 5 | 28 | 32 | 23 | 2 | 19 |
| Kiroba | 33 | 20 | 4 | 19 | 28 | 16 | 2 | 15 |
| Albert | 22 | 17 | 2 | 14 | 16 | 9 | 0 | 8 |
| Mean[c] % | 32 | 25 | 11 | | 25 | 16 | 1 | |

[a]Cuttings grown from CBSD-infected mother plants for 6 months without CBSD symptoms were further tested by RT-PCR and plants that tested negative were considered reverted (virus-free).
[b]Mean (%) plants that were tested negative by RT-PCR in the three cassava varieties across different cuttings length.
[c]Mean (%) plants that were tested negative by RT-PCR in different cuttings across the three cassava varieties.

### 6.3.6 Effect of stem position (lower, middle and upper portions) on reversion

Reversion was greatest in cuttings taken from the upper and middle parts of the stem compared to the lower part (Figure 6.18), especially for UCBSV-[UG:Kab4-3:07]-infected cuttings. Overall, reversion in cuttings from CBSV-[MZ:Nam1-1:07] infection range from 2% in the lower stems of Albert to 38% from the middle stems of Kaleso. For UCBSV-[UG:Kab4-3:07], it ranged from 5% in the lower stems of Albert to 43% from the upper stems of Kaleso (Figure 6.18).

Significant differences among cassava varieties ($P<0.001$) and virus isolates ($P<0.0094$) were observed in reversion to CBSD in the three cassava varieties. The effect of the stem positions at which cuttings were taken (lower, middle and upper) across the three cassava varieties was also significant ($P<0.01$). Albert differed significantly from Kaleso and Kiroba for reversion from cutting position ($P<0.001$), but no significant differences were observed between Kaleso and Kiroba ($P>0.7$). Likewise there were significant differences between middle and lower stem ($P<0.008$) and also between upper and lower stem ($P<0.02$), but no significant differences ($P>0.9$) between upper and middle stem.

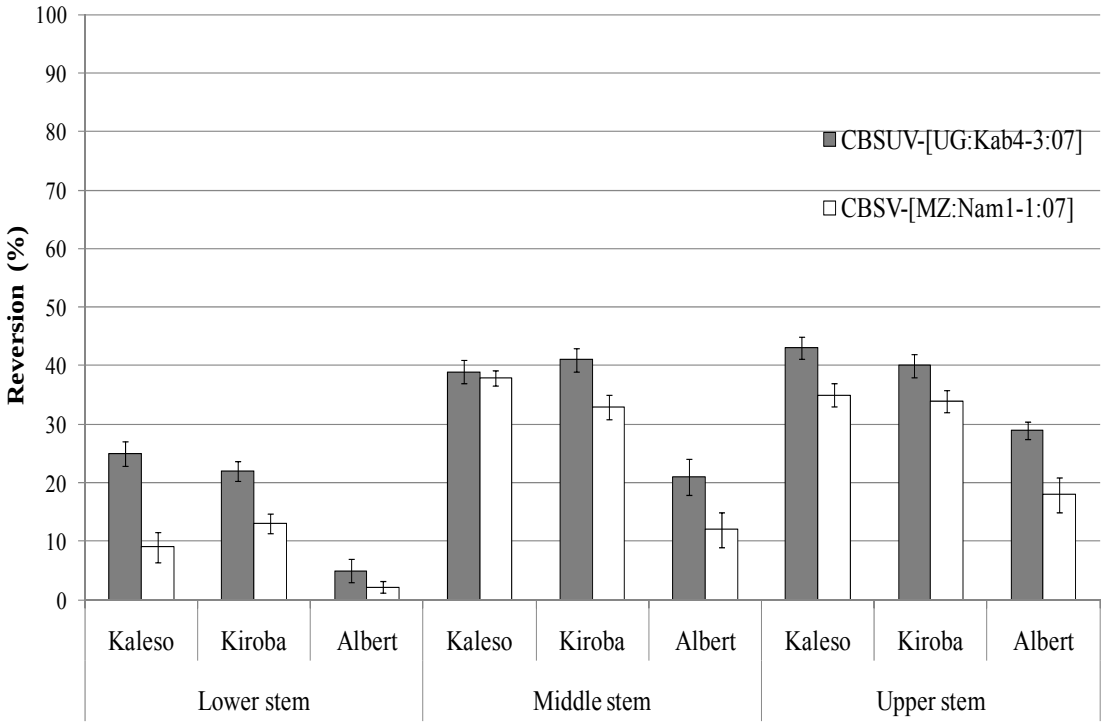



**Figure 6.18**: Effects of isolate and variety on the rate of reversion for cuttings taken from smaller, middle and upper parts of the stems of three cassava varieties.

### 6.3.7 Fecundity and survival of *B. tabaci* on cassava varieties

All three cassava varieties supported the reproduction of whitefly equally (Figure 6.19). Minor differences observed in the numbers of eggs laid, nymphs developed and adults emerged on each variety were not statistically significant between the three cassava varieties. The variety Albert supported a greater number of eggs (319) and nymphs (288) than Kiroba (304), (273) and Kaleso (316), (286) respectively. Adult eclusion was favoured by varieties Kaleso (262) and Albert (261) than Kiroba (252) (Figure 6.19). But these differences were not statistically significant when an ANOVA test was carried out on the data.

The differences in mean development time for *B. tabaci* among cassava varieties for eggs to nymphs, nymphs to adults and eggs to adults were also not significant ($P > 0.05$). The percentage eggs that survived to nymphs across varieties ranged from 90-91%, nymphs to adults were 91-92% and eggs to adults were 82-84% (Figure 6.20). The greatest percentage survival from eggs to adults was observed on Kaleso (84%); followed by Kiroba (83%), while the lowest survival from eggs to adults was observed on Albert.

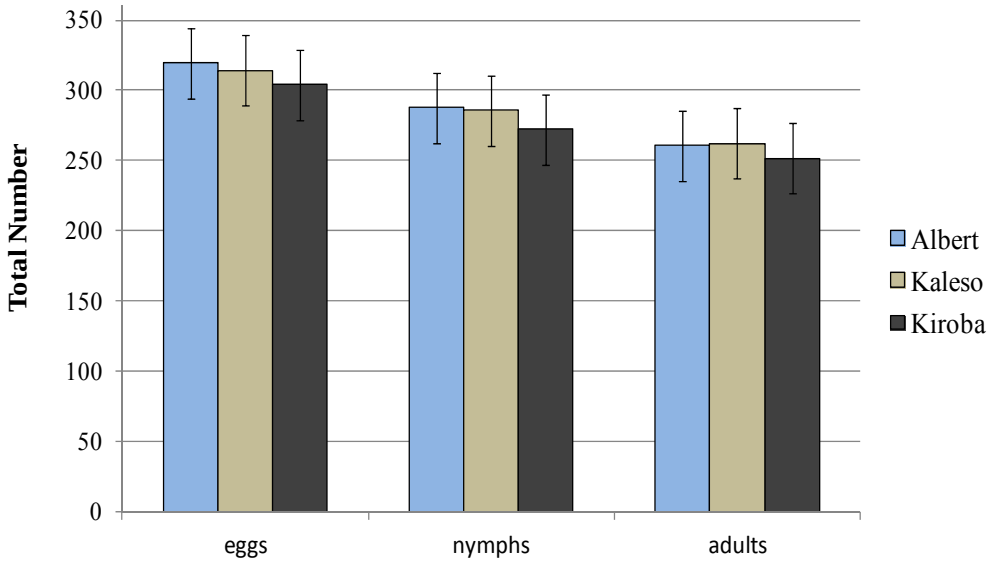



**Figure 6.19**: Total number of eggs, nymphs and adult *B. tabaci* recorded on the three cassava varieties.

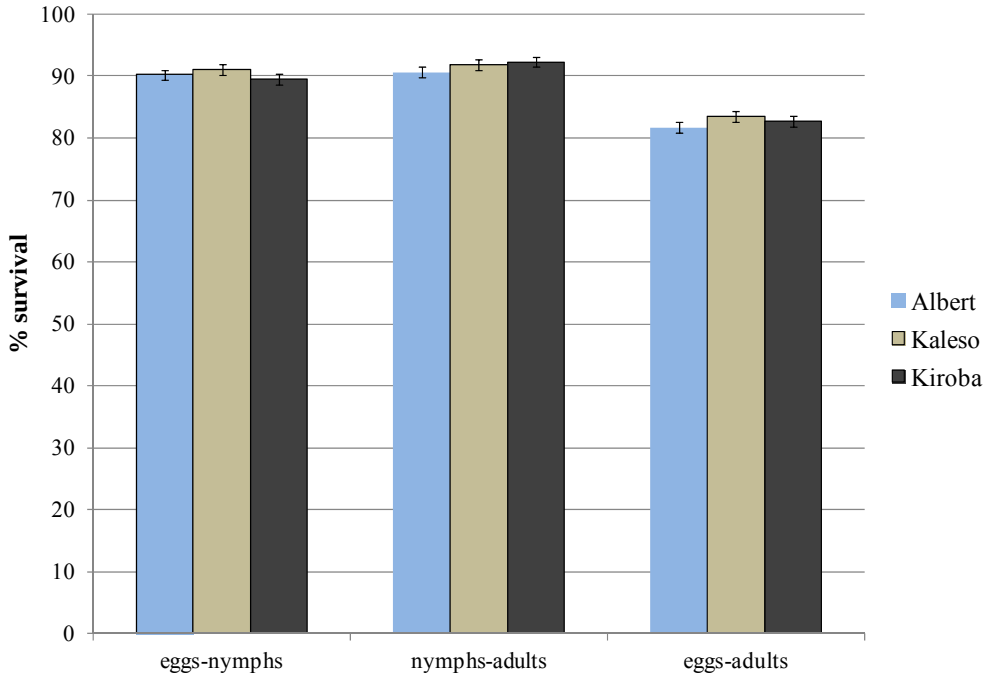



**Figure 6.20**: Development of *B. tabaci* on three cassava varieties. Bars represent percent number and survival of *B. tabaci* on cassava var. Albert, Kaleso and Kiroba.

### 6.3.8 Resistance/susceptibility of cassava varieties to CBSV upon transmission by *B. tabaci*

The overall transmission rate recorded was 36% across all three varieties tested. The greatest transmission rate was recorded in var. Albert 57%, followed by Kiroba (47%). Kaleso was the most resistant variety with infection recorded on one plant (3%) (Table 6.8). The differences among cassava varieties for the rate of CBSV transmission were highly significant ($P<0.001$). The number of weeks required from inoculation to symptom appearance varied; in Albert the first plant showed symptoms three weeks after inoculation while in Kiroba and Kaleso, first symptoms appeared five and eight weeks after inoculation, respectively. Symptoms in vector-transmitted plants were similar to those seen in plants obtained from CBSV-infected cuttings and graft-inoculated plants.

Table 6.8: Rate of CBSV transmission in three different cassava varieties and number of CBSV-infected plants detect by RT-PCR.

| Variety | Experiment 1 | Experiment 2 | Experiment 3 | | |
|---|---|---|---|---|---|
| | CBSV positive/n[d] | CBSV positive/n[d] | CBSV positive/n[d] | Total | % infected plants[a] |
| Kaleso | 1/10 | 0/10 | 0/10 | 1/30 | 3 |
| Kiroba | 6/10 | 5/10 | 3/10 | 14/30 | 47 |
| Albert | 6/10 | 6/10 | 5/10 | 17/30 | 57 |
| Total | 13/30 | 11/30 | 8/30 | 32/90 | |
| % infected plants[b] | 43 | 37 | 27 | 36 | 36[c] |

[a]Average rate of transmission recorded in each cassava variety
[b]Average rate of transmission in each experiment across the varieties
[c]Average rate (%) of transmission in 90 cassava plants used in CBSV transmission experiments.
[d]Number of plants with CBSV/Number used for whitefly transmission in each experiment.

### 6.3.9 Relationship between visual observations of CBSD-symptoms and CBSV detection in *B. tabaci* inoculated cassava plants

The relationship between the observation of CBSD foliar symptoms and presence of the virus was established for the above 90 samples inoculated by *B. tabaci*. The var. Albert had most of CBSV-infected plants with chlorotic spots, followed by

Kiroba and Kaleso, (Appendix 1.4). In the first experiment, 60% plants each of Albert and Kiroba, and 10% of Kaleso, produced typical CBSV symptoms. In the second experiment, the rate of infection was similar on Albert and Kiroba but Kaleso was not infected. A smaller rate of infection was recorded in the third experiment, in which 50% of Albert were CBSV positive, while only 30% were infected in the Kiroba (Appendix 1.5). There was a positive relationship between visual observation of symptoms in all the three varieties and detection of CBSV by RT-PCR, except for one or two cases in Kiroba (Appendix 1.6).

### 6.3.10 Classification of cassava varieties into different resistance groups

The three cassava varieties were tested with UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] and a number of parameters including the severity of symptoms, disease incidence in leaves, stems, roots, virus replication and movement was analysed by RT-PCR and virus titres were recorded based on these parameters, none of the varieties had complete immunity to CBSV-[MZ:Nam1-1:07], but Kaleso was found to have a greater level of resistance, while Kiroba was tolerant to CBSV-[MZ:Nam1-1:07], but had moderate resistance to UCBSV-[UG:Kab4-3:07] and Albert was most susceptible (Table 6.9).

Table 6.9: Summarised tabular form of the resistance mechanisms.

| Parameters[a] | UCBSV-[UG:Kab4-3:07] | | | CBSV-[MZ:Nam1-1:07] | | |
|---|---|---|---|---|---|---|
| | Kaleso | Kiroba | Albert | Kaleso | Kiroba | Albert |
| Graft-inoculation | MR | MR | S | R | T | HS |
| Leaf incidence | MR | MR | S | R | T | HS |
| Stem incidence | HR | MR | S | R | T | HS |
| Leaf severity | MR | MR | S | R | T | HS |
| Root necrosis | HR | MR | S | R | T | HS |
| Virus replication | HR | MR | S | R | T | HS |
| Virus titre | HR | MR | S | R | T | HS |
| Reversion | MR | MR | S | R | T | HS |
| *B. tabaci* fecundity | HS | HS | HS | HS | HS | HS |
| *B.tabaci*-inoculation | NA | NA | NA | HR | S | HS |

[a]Resistance status of cassava varieties based on their reactions to infections with UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] by graft-inoculation. CBSVs infection based on the number of plants per isolate that developed CBSD leaf, stem and root symptoms after graft-inoculation, CBSD symptom severity score, virus spread within cassava varieties and high virus load. Varieties were classified as: S = susceptible, HS = highly susceptible, T = tolerant, MR = moderately resistant, R = resistance, HR = highly resistant and NA = not assessed.

## 6.4. Discussion

This study was initiated to investigate the interactions between two CBSD isolates with their cassava host, to improve our understanding of the mechanisms of resistance to CBSD. All three cassava varieties (Kaleso field-resistant, Kiroba tolerant and Albert susceptible) were infected by graft-inoculation. Clear UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] infections similar to those seen earlier (Winter *et al*., 2010) were obtained but the rate of transmission and the time it took to express symptoms varied between varieties (Table 6.4). Grafting was highly effective for screening cassava for CBSD resistance and to differentiate between resistant and susceptible cassava varieties. The reaction of the varieties to infection by graft-inoculation varied with the number of grafts and time to the first appearance of symptoms. Albert became infected with both viruses with one graft-inoculation, while more than one grafting was necessary to infect Kaleso and Kiroba and symptom expression was delayed. These observations were consistent with earlier reports that resistant cassava varieties are known to suppress CMV multiplication and movement in infected plants (Thresh and Cooter, 2005).

The findings of this study were also similar to the results obtained in earlier studies in which all cassava varieties selected for resistance in the field became infected when graft-inoculated (Storey, 1947; Ogbe *et al*., 2002). This may be because the normal mechanism of vector transmission was bypassed when high virus titres were inoculated with grafting. Similar observations have been made on begomoviruses in which resistance was lost when an infected scion was grafted on resistant tomato plants (Vidavsky and Czosnek, 1998). In grafting, the virus was delivered directly into the vascular system continually for as long as the scion remains viable, thus suppresses resistance mechanisms (Kheyr-Pour *et al*., 1994). Also important to note is that the graft-inoculated scion was derived from a susceptible cassava variety, which provides a reservoir on the inoculated plant in which virus replication might continue regardless of the resistance of the stock plants. Such conditions of introducing high viral inoculums do not exist with whitefly transmitting the viruses. With *B. tabaci* transmission, success of infection depends on successful replication and translocation of the few virus particles ingested during vector feeding.

The symptom type and the time interval between graft-inoculation and symptoms appearance depended on the variety. Symptom development was delayed significantly on Kiroba and Kaleso compared to Albert, which was consistent with field observations (Hillocks *et al*., 2001; Hillocks, 2003). This varietal difference was previously described as due to the restricted movement of virus on resistant cassava varieties (Walkey, 1985). Although such evidence is lacking for CBSD, Thottappilly *et al*. (2003) described six types of resistance mechanisms to CMD in cassava; resistance to inoculation, field vector resistance, virus titre resistance, symptom severity, resistance to virus spread and development of symptoms over time. In this study, we used field-resistant, tolerant and susceptible cassava varieties, which enabled us to observe the differences in response of different varieties to infection by UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] over a long period of 96 weeks. Our data on the detection of viruses in leaves and roots indicated that resistance of cassava to UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] can be manifested in the restriction of virus movement as well as the suppression of virus multiplication in Kaleso. The findings of this study are in agreement with the earlier study on ACMV, which was incompletely systemic in resistant cassava (Rossel *et al.,* 1992; 1994; Njock *et al.,* 1996).

We have followed the distribution of UCBSV-[UG: Kab4-3:07] and CBSV-[MZ: Nam1-1:07] in graft-inoculated Kaleso, Kiroba and Albert. Early detection of CBSV in roots compared to shoots is consistent with the classical study on the nature of virus movement in plants as demonstrated by Samuel (1934) with *Tobacco mosaic virus* (TMV). TMV particles were shown to be systemically translocated through phloem to lower parts of the tomato plant and subsequently re-distributed to the youngest leaves and the rest of plant shoots. Jennings (1960a) observed that virus introduced to upper part of the plants can move down to infect roots even in resistant plant. This downward movement was confirmed in graft-inoculation experiment. The interaction of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] with cassava varieties in CBSD-pathosystem can be seen as molecular arms race between the virus and host defence mechanisms. These interactions were reported to have depended on the immune systems of the host (Boevink and Oparka, 2005) and might explain the reason why cassava varieties

differ greatly in CBSD symptom development, severity, virus movement and titre, even when infection occurs at the same time.

UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] have different pathogenicities on cassava varieties. Kiroba was tolerant to both viruses. Although Kaleso can be infected by both UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07], the variety does not show severe symptoms, while Albert is susceptible. The qPCR results are consistent with recent studies that reported correlation between symptom severity and virus titres in CBSV (Moreno *et al.,* 2011). In our studies Albert infected with CBSV-[MZ:Nam1-1:07] expressed severe symptoms and showed high virus titre. UCBSV-[UG: Kab4-3:07] produced milder symptoms on Albert, which was reflected in the relatively low virus titre. A similar effect was observed in all three cassava varieties, where the titres of milder UCBSV-[UG: Kab4-3:07] was smaller than the severe CBSV-[MZ: Nam1-1:07]. The observed decline in virus titre in resistant cassava varieties was suggested to be due to reversion phenomenon by Van den Bosch *et al*. (2007).

An obvious advantage of this information will be for screening cassava varieties for virus resistance by measuring virus titre in leaves together with an assessment of symptoms. If reduced viral replication in leaves can be correlated to the absence of severe symptoms, the RT-qPCR could be used as an effective tool for screening for CBSD resistance. Our results reported for the first time the effect of resistance mechanisms in restricting virus replication and spread.

The greatest proportion of disease-free plants was obtained (reversion) from the resistant variety Kaleso. These results extend previous findings for CMBs that reversion is more likely to occur in resistant plants than in susceptible varieties (Jennings, 1960b; Rossel *et al*., 1992; Thresh *et al*., 1994; Fargette *et al*., 1996). Fondong *et al.* (2000) observed reversion in CMD-affected plants Gibson and Otim-Nape (1997) also confirmed reversion occurring in CMD-resistant var. TMS 30572, but not in the susceptible Bao. For CBSD, reversion was observed in our studies in the susceptible var. Albert, albeit at a smaller rate than the tolerant (Kiroba) and resistant (Kaleso). The virulence levels of the viruses are also

believed to play an important role in reversion. Studies on CMD have associated reversion with both mild and severe forms of the virus (Gibson and Otim-Nape, 1997; Pita *et al*., 2001). A similar observation was made in this study, where CBSD-affected stem cuttings from the severe CBSV-[MZ:Nam1-1:07] produced less disease-free plants and a smaller percentage reversion compared to the mild UCBSV-[UG:Kab4-3:07]. Storey (1938) noted that some cassava cuttings taken from CBSD-affected plants sprouted without symptoms. He also observed differences in symptom severity of different varieties affected by CBSD and referred to them as due to distinct strains. Cassava varieties differed in the expression of CBSD symptoms, and one reason could be a difference in their ability to revert as result of the differences in the ability of the viruses to suppress posttranscriptional gene silencing in different cassava plants. For instance, amino acid substitutions in HC-Pro lead to less titres of *Potato virus A* of the genus *Potyvirus* in tobacco leaves leading to reversion in most of the plants (Andrejeva *et al*., 1999). Reversion was earlier reported to be as result of RNA silencing mechanism for CMD (Fondong *et al*., 2000). It is therefore very likely that the reversion observed for CBSD is also due to RNA silencing, which will have to be verified in future studies.

The greatest number of virus-free plants was grown from middle and upper portions of CBSD-affected cuttings compared to lower part of the stems. However, high mortality occurred in the cuttings taken from the upper part of the plants which could be due to the tenderness of cuttings from this part of the stems. Nevertheless, the findings of this study are in agreement with the earlier suggestion of Hillocks and Jennings (2003) that CBSV distribution in cassava could be localised such that cuttings taken from a certain part of infected plants could become symptomless and sprout without symptoms. In areas of high CBSD incidence, farmers can be encouraged to take planting material only from the middle and upper parts of the cassava stems to exploit the inherent mechanism of reversion from virus infection by cassava plants. Taking planting material from middle part of the stems can minimise the risk of poor crop establishment that was earlier associated with cuttings taken from the upper part of the stems (Gibson and Otim-Nape, 1997). The length of cuttings also affected both plant regeneration and reversion in CMD (Fondong *et al*., 2000). Similarly, in this

study, short cuttings of 10 cm produced most number of virus-free plants than longer ones (15 and 20 cm), although overall plant regeneration was high in longer cuttings. The general trend indicated that the smaller the length of the cuttings, the greater the probability of producing CBSD-free plants of Kaleso, Kiroba and Albert.

The findings of the study on the fecundity and survival of *B. tabaci* on the three cassava varieties are in agreement with an earlier report which pointed to the lack of differences in the fecundity of whiteflies on cassava varieties (Maruthi *et al*., 2001). Hahn *et al*. (1980) similarly observed no differences in *B. tabaci* survival on CMD-resistant, tolerant and susceptible cassava varieties and thus concluded that resistance to the vector was unlikely in cassava, although Fargette *et al*. (1996) indicated whitefly survival differed considerably between varieties in the field. Results obtained in this study provide no evidence of differences between the resistant, tolerant and susceptible cassava varieties to support whitefly survival and reproduction. Our results did not provide a link between the mechanisms of resistance to CBSV to unattractiveness of *B. tabaci* in these varieties. These results however, provide a basis for comparing the rate of CBSV transmission by whiteflies. The results of the fecundity study further support the view that resistant features manifested by some cassava varieties are not affected by the fecundity of *B. tabaci* on cassava, which supports the conclusions that the resistance/tolerance to CBSV found in cassava varieties are due to the inherent property of the varieties to the virus.

The transmission rates achieved in this study were high (up to 57%) compared to the ones reported previously (22%) (Maruthi *et al*., 2005) and (28%) (Mware *et al*., 2009). This could be due to the improvement in the transmission protocols followed such as allowing the whiteflies to acquire the virus freely on a diseased plant and using a set AAP and IAP of 24 h. Environmental conditions and feeding behaviour of adult *B. tabaci* on cassava plants may adversely affect transmission (Maruthi *et al*., 2005). For instance, in transmission with spiralling whitefly, high humidity within the clip cages lead to mass mortality of spiraling whitefly, but *B. tabaci* was able to survive humid conditions (Mware *et al*., 2009). The effect of the environment was eliminated in this study as similar conditions were

maintained throughout the technical updates. High transmission of CBSV by the *B. tabaci* was obtained in the field on susceptible and tolerant varieties, compared to the resistant varieties (Mware *et al*., 2009). High *B. tabaci* populations in the fields may be correlated with the high CBSD incidences as was observed in Uganda (Alicai *et al*., 2007). This may explain the reason for the possible outbreak of CBSD in cassava growing areas in high coastal areas of the country. Management options need to focus on the control of the *B. tabaci* (vector), propagation of cassava varieties that are resistant to UCBSV and CBSV infection, in addition to other control measures. Kaleso which was identified in this study can be one of such variety, since it was proved to be resistant to virus replication.

### 6.4.1 Conclusions

The main conclusions arising from Chapter 6 are:

1- Development of CBSD symptoms over time is variety and isolate dependent. CBSV-[MZ:Nam1-1:07] was severe on cassava compared to UCBSV-[UG:Kab4-3:07], which is mild. Symptoms on leaves are associated with root symptom in Albert infected with both viruses. In Kaleso and Kiroba, foliar chlorosis did not associate well with root necrosis, particularly for UCBSV-[UG:Kab4-3:07] since they had no or limited root symptoms, suggesting that root and leaf symptoms can occur independently at least for some virus-variety combinations.

2- The resistance mechanisms that prevent or slow down the movement of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] following graft-inoculation is dependent upon the tolerance level of the varieties. In resistant cassava varieties, virus replication and titre are suppressed leading to recovery from symptoms and virus. Reversion is observed for CBSD in cassava and it depends on the levels of resistance/tolerance of the varieties.

3-The length of cuttings also affected plant regeneration and reversion in CBSD; the smaller the length of the cuttings, the greater the CBSD-free plants.

4- For the cassava varieties used in this study, susceptibility to CBSD cannot be attributed to differences in their ability to support *B. tabaci*. Resistance levels observed therefore are for the virus.

# CHAPTER 7: Developing methods to eliminate UCBSV and CBSV from infected cassava varieties

## 7.1. Introduction

Elimination of virus from cassava by tissue culture was first described by Morel and Martin (1952), by thermotherapy (Nyland and Goheen, 1969) and chemotherapy (Quak, 1961) while tissue culture alone was found to be sufficient for removing CMBs from cassava (Roca *et al*., 1984; Kartha, 1981). Cassava varieties with complete resistant to UCBSV and CBSV are not known and demands for healthy and certified planting materials have recently been increased in SSA. Alternative ways of generating virus-free cassava therefore offers a way of controlling CBSD. Development of an efficient virus eradication technique for UCBSV and CBSV from cassava infected plants is also critical in quarantine and germplasm collections in SSA.

Chemo and thermotherapies have been effective in eliminating several viruses known to infect vegetatively propagated crops (Allam, 2000; Nascimento *et al.*, 2003). Recently, Wasswa *et al*. (2010) demonstrated that CBSV elimination in cassava could be achieved by a combination of tissue culture and heat therapy. These methods however, have not been developed for the cassava varieties with different tolerant levels to CBSD and for both UCBSV and CBSV. The aim of the study was to develop methods to eliminate UCBSV and CBSV from infected cassava varieties. The therapies were then compared for their efficiency on plant regeneration and the elimination of viruses.

## 7.2. Materials and methods

### 7.2.1 Cassava varieties and CBSD isolates

Cassava plants of Kaleso, Kiroba and Albert that tested positive for UCBSV-[UG:Kab4-3:07] or CBSV-[MZ:Nam1-1:07] by RT-PCR were selected (see below).

### 7.2.2 Tissue culturing

Fifty single node cuttings from young stems of each of the three cassava varieties were excised (~0.4 mm) and surface sterilised (section 3.2.2). The nodes were cultured on basal medium (Murashige and Skoog, 1962), which was modified in this study (see section 3.2.1). The plantlets were grown in a constant environment for eight weeks, and then transferred into the soil and weaned as described in Chapter 3 (section 3.2.3). The tissue culture experiments were repeated three times using 50 nodal cuttings for cassava regeneration and virus elimination from each variety (Kaleso, Kiroba and Albert) and virus. Twenty healthy nodal cuttings from each cassava variety were inoculated into the tissue culture media and used as controls for each set up.

### 7.2.3 Comparison of the position of the nodes for virus elimination

Nodes from each plant were numbered 1-10 from top to bottom and classified into two categories for easy comparison as top (node numbers 1–5) and bottom (node numbers 6–10). Nodes were surface sterilised as described previously (section 3.2.2) and then transferred to their respective media and grown in the tissue culture room. Tissue-cultured plantlets were scored for the presence/absence of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] using visual observations and tested by RT-PCR using virus-specific primers (CBSV F3 and CBSV R3). Plants that showed disease symptoms after three months were discarded, and symptom-free plants were allowed to grow for six months and then tested by RT-PCR.

### 7.2.4 Thermotherapy

The thermotherapy experiment was a modification of protocol used for the production of virus-free plants from yam (Balagne, 1985). Ten node cuttings each from UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-infected plants of vars. Kaleso, Kiroba and Albert were excised (~0.4 mm), inoculated into the tubes containing supplemented MS media (section 3.2.1). The plantlets were kept in the incubator (Leec, UK) at different temperature regimes of 30, 35, 40 and 45 $^{o}$C for three weeks with 12 h of light and 12 h of darkness (L12:D12). Plantlet survival was recorded from each temperature regime for each variety-virus combination. After three weeks, the plantlets were removed from the incubator

and transferred into a tissue culture growth room for one week and then planted in pots (0.5 litre) filled with steam sterilised compost:soil (1:1) in a quarantine glasshouse. Plants were grown under propagator lids and the tending period (section 3.2.3) was increased from 2 to 3–4 weeks to reduce plant mortality. Presence or absence of CBSD symptoms was recorded monthly by visual observation of treated plants. After six months, leaf samples were collected from plants that were not showing CBSD symptoms and tested for viruses using RT-PCR. The experiment was repeated three times using 10 nodal cuttings for each variety-virus combination and four temperature regimes. Twenty healthy nodal cuttings were inoculated into the tissue culture media for each temperature regime per variety as control for each set up.

### 7.2.5 Chemotherapy

The antiviral chemical ribavirin (1,ß-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide) (Sigma R9644), (Scientific Laboratory, UK) supplied in powder form was tested for its efficiency for the elimination of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] from three cassava varieties. Ribavirin has broad spectrum anti-viral activities (Dawson, 1984; Fletcher *et al*., 1998). Three concentrations (15 mg/l = 0.06 mM/l), (25 mg/l = 0.1 mM/l) and (50 mg/l = 0.21 mM/l) of ribavirin were tested and control media was made containing no ribavirin. Ribavirin is a toxic compound so extreme care was taken when handling it.

Chemotherapy was carried out on nodes from five plants of the three cassava varieties for the two viruses; UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. Sterile single use plastic tubes (25 ml size, Sterilin, UK) were used since ribavirin is toxic and the contaminated tubes could be disposed of after use by incineration without the need for lengthy decontamination procedures. Fifty nodes per variety for each treatment were transferred to glass tubes containing the media supplemented with ribavirin. The experiment was repeated three times using 50 nodes from each variety-virus combinations and for three ribavirin concentrations. Fifty healthy nodal cuttings per variety were inoculated into the tissue culture media without ribavirin to use as control for each set up.

### 7.2.6 Simultaneous application of the therapies for *in vitro* regeneration of cassava and UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]

Chemo and thermotherapy were carried out on tissue cultured plants of the three cassava varieties from UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-infected plants. Thirty nodes per variety were transferred to glass tubes containing the media supplemented with ribavirin at 0.1 mM/l concentration. The plantlets were kept in the incubator (Leec, UK) at 40 $^{o}$C for three weeks with L12:D12. Plantlet survival was recorded from each variety-virus combination. After three weeks, the plantlets were removed from the incubator and transferred into a tissue culture growth room for one week and then planted in pots (section 7.2.4) in a quarantine glasshouse. Plants were grown under propagator lids for 3–4 weeks. Presence or absence of CBSD symptoms was recorded monthly by visual observation of treated plants. After six months, leaf samples were collected from plants that were not showing CBSD symptoms and tested for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] using RT-PCR. The experiment was repeated three times. Twenty healthy nodal cuttings per variety were cultured into the media containing ribavirin and exposed to the same temperature regime to use as control for each set up.

### 7.2.7 Assessment of the efficacy of the therapies

All the plantlets resulting from the therapy trials outlined above were tested for presence or absence of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] at six months after treatment to allow for proper plants growt. The RNA extraction protocol described in Chapter 3 (section 3.2.7) was used followed by RT-PCR using virus specific primers CBSV F3 and CBSV R3 as described in chapter 3 (section 3.2.8 and 3.2.9). In each trial, the number of plantlets regenerated over the total number inoculated was indicated in tables. The efficiency of the therapy (ET) was determined using the formula described previously (Hormozi-Nejad *et al.*, 2010; Mahfouze *et al.*, 2010; Meybodi *et al.*, 2011). The equation used was: % ET = % plant regenerated x % virus-free plants / 100. The ET of tissue culture alone, thermotherapy, chemotherapy and Simultaneous application of the treatments were compared for their efficacy at eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] from *in vitro* cassava plants.

## 7.3 Results

### 7.3.1 Tissue culturing

Elongation of auxillary buds and emergence of new leaves were observed two weeks after seeding of the explants (Figure 7.1a) while root formation took three weeks (Figure 7.1b). About 4-6 weeks after inoculations, plantlets were ready for transfer into soil (Figure 7.1c); they were further tendered for 3-4 weeks and observed monthly for 6 months. Dead and CBSD-affected plants were removed monthly (Figure 7.1e and f). In general greater number of virus-free plants were recorded from UCBSV-[UG:Kab4-3:07]-infected plants of the three varieties compared to CBSV-[MZ:Nam1-1:07] (Table 7.1). All surviving plants that remained symptom-free after six months were also shown to be virus-free by RT-PCR. These data were used for the therapy efficiency analysis in section 7.3.6.


**Figure 7.1**: Regeneration of CBSD-infected node cuttings from cassava varieties by tissue culture for eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. (a) Explants one week after inoculation into tissue culture media, plantlets at four weeks after inoculation (b), plantlets six months after been transferred into soil (c), plantlets two months in the soil (d), virus-free plants (e) and CBSV-[MZ:Nam1-1:07]-positive (red arrows) plants (f)

Table 7.1: Effect of tissue culture in eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] from infected cassava varieties.

| | Number of virus-free plants by RT-PCR/Number tested[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | | | | CBSV-[MZ:Nam1-1:07] | | | | | |
| Tissue culture | Kaleso | | Kiroba | | Albert | | Kaleso | | Kiroba | | Albert | |
| Nodes[a] | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f |
| No1 | 0/15 | nt | 6/15 | 6/6 | 6/15 | 2/6 | 6/15 | 3/6 | 7/15 | 5/7 | 6/15 | 2/6 |
| No2 | 10/15 | 6/10 | 12/15 | 9/12 | 9/15 | 3/9 | 10/15 | 4/10 | 10/15 | 4/10 | 9/15 | 3/9 |
| No3 | 10/15 | 8/10 | 10/15 | 4/10 | 10/15 | 1/10 | 9/15 | 2/9 | 12/15 | 3/12 | 11/15 | 2/11 |
| No4 | 6/15 | 4/6 | 9/15 | 5/9 | 8/15 | 2/8 | 10/15 | 4/10 | 12/15 | 6/12 | 9/15 | 1/9 |
| No5 | 1/15 | 1/1 | 11/15 | 4/11 | 8/15 | 4/8 | 6/15 | 2/6 | 7/15 | 3/7 | 9/15 | 2/9 |
| No6 | 9/15 | 3/9 | 12/15 | 3/12 | 8/15 | 0/8 | 8/15 | 0/8 | 9/15 | 1/9 | 11/15 | 3/11 |
| No7 | 8/15 | 8/9 | 8/15 | 3/8 | 7/15 | 0/7 | 7/15 | 0/7 | 8/15 | 1/8 | 9/15 | 0/9 |
| No8 | 10/15 | 2/10 | 10/15 | 2/10 | 10/15 | 1/10 | 9/15 | 0/9 | 11/15 | 2/11 | 9/15 | 0/9 |
| No9 | 14/15 | 5/14 | 13/15 | 4/13 | 12/15 | 3/12 | 9/15 | 0/9 | 9/15 | 0/9 | 8/15 | 0/8 |
| No10 | 8/15 | 0/8 | 13/15 | 5/13 | 8/15 | 0/8 | 8/15 | 0/8 | 8/15 | 0/8 | 9/15 | 0/9 |
| Mean | 76/150 | 37/76 | 104/150 | 45/104 | 86/150 | 16/86 | 82/150 | 15/82 | 93/150 | 25/93 | 90/150 | 13/90 |

[a]15 nodes were planted into tissue culture media from each position. Results were recorded as number regenerated (Reg) per inoculated, the number of virus free (V.f) plants.

### 7.3.2 Comparison of the position of nodes as material for starting explants

About 71% of nodes from the bottom of the plant (from positions 6 to 10) grown in the tissue culture media survived whereas only 54% nodes from top position (from positions 1 to 5) survived using our protocol. Contamination of the nodes cutting recorded in the media was greater from lower positions (19%) than from the upper (5%). Similarly, percentage node cuttings grown into green state was greater from the lower position than from the upper position. However, the percentage nodes that died in the media were greater from the upper (24%) than from the lower part of the plants (Table 7.2).

Table 7.2: Comparison of the survival of cassava nodes in tissue culture media four weeks after been grown on the media.

| Tissue culture plants that survived in both media and after transferred in soil | | | | | Number |
|---|---|---|---|---|---|
| Plant part | Contaminated % | Dead % | Green state[a] % | Growth[b] % | in soil[c] % |
| Upper (nodes 1-5) | 5 | 24 | 71 | 62 | 54 |
| lower (nodes 6-10) | 19 | 5 | 77 | 75 | 71 |

[a]Nodes that were still green or callus formation were all considered as "green state"
[b]Growth include roots, stems and/or leaf formation, but sometimes the developed tissue was insufficient for virus-testing purposes.
[c]Nodes that developed into fully grown plants after transfer into soil, including those of the control treatment.

### 7.3.3 Thermotherapy

Cassava plantlets showed varying responses to heat treatment. Heat stress ranged from singed leaves and shoot tips (at 40 $^{o}$C) to total death of the plantlets in the three cassava varieties especially at the greatest temperature (45 $^{o}$C) while 30 $^{o}$C and 35 $^{o}$C appeared to have a positive effect on plant growth and development (Figure 7.2 and Table 7.3). Greater number of virus-free plants was obtained at 40 $^{o}$C treatment compared to 30 $^{o}$C and 35 $^{o}$C (Table 7.3). More virus-free plants were obtained from UCBSV-[UG:Kab4-3:07]-infected node cuttings than from CBSV-[MZ:Nam1-1:07] (Table 7.3). These data were used in the efficiency of the therapy analysis in section 7.3.6.

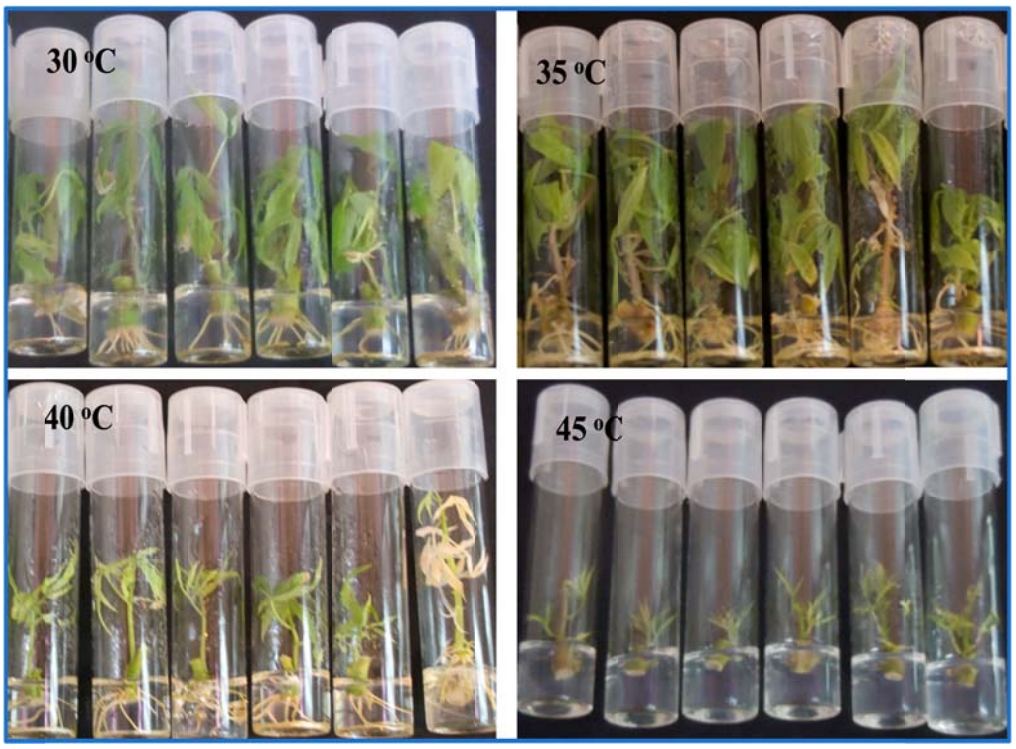



**Figure 7.2:** Regeneration of CBSD-infected node cuttings from cassava varieties by thermotherapy at different temperature regimes. Inoculated nodes were kept in the incubator (Leec, UK) for three weeks at L12:D12 for eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07].

Table 7.3: Effect of thermotherapy on UCBSV-[UG:Kab4-3:07] and CBSV[MZ:Nam1-1:07]-elimination from infected cassava varieties.

| Thermo therapy | Number of virus-free plants viruses free by RT-PCR/Number tested[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | | | | CBSV-[MZ:Nam1-1:07] | | | | | |
| | Kaleso | | Kiroba | | Albert | | Kaleso | | Kiroba | | Albert | |
| Temp.$^{o}$C | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f |
| 30 | 21/30 | 11/21 | 26/30 | 18/26 | 22/30 | 9/22 | 26/30 | 12/26 | 28/30 | 15/28 | 24/30 | 5/24 |
| 35 | 28/30 | 17/28 | 28/30 | 25/28 | 25/30 | 11/25 | 27/30 | 19/27 | 27/30 | 21/27 | 29/30 | 16/29 |
| 40 | 14/30 | 13/14 | 16/30 | 16/16 | 16/30 | 12/16 | 10/30 | 8/10 | 19/30 | 18/19 | 15/30 | 11/15 |
| 45 | 0/30 | nt | 0/30 | nt | 0/30 | nt | 0/30 | nt | 0/30 | nt | 0/30 | nt |
| Mean | 63/120 | 41/63 | 70/120 | 59/70 | 63/120 | 32/63 | 63/120 | 39/63 | 74/120 | 54/74 | 68/120 | 32/68 |

[a]Nodes were inoculated into tissue culture media for each variety-virus combination and transferred to the incubator (Leec, UK) for 3 weeks at L12:D12 for each temperature regime (Temp.$^{o}$C). Results were recorded as number regenerated (Reg.) per number inoculated, number virus free (V.f) per number regenerated, nodes not tested (nt) due to contamination or dead.

### 7.3.4 Chemotherapy

Phytotoxic effects of the ribavirin at the greatest concentration of 0.21 mM/l was observed which resulted in severe stunting of plantlets, thin stems, stunted leaflets, sluggish root development and finally death of all the plantlets in all three cassava varieties (Figure 7.3). The development of roots was sluggish also (at 0.1 mM/l ribavirin). Greater number of virus-free plants was obtained from 0.1 mM/l treatment compared to 0.06 mM/l (Table 7.4). Plantlets regenerated after exposure to chemotherapy at 0.06 mM/l of ribavirin were morphologically identical to those regenerated from non-treated control plants (Figure 7.4).

The number of regenerated plantlets after growing on media supplemented with ribavirin were greater from UCBSV-[UG:Kab4-3:07]-infected node cuttings than CBSV-[MZ:Nam1-1:07]. Similarly, more virus-free plants were obtained from UCBSV-[UG:Kab4-3:07]-affected plants compared to CBSV-[MZ:Nam1-1:07] (Table 7.4). These data were also used to estimate the efficiency of chemotherapy (section 7.3.6).

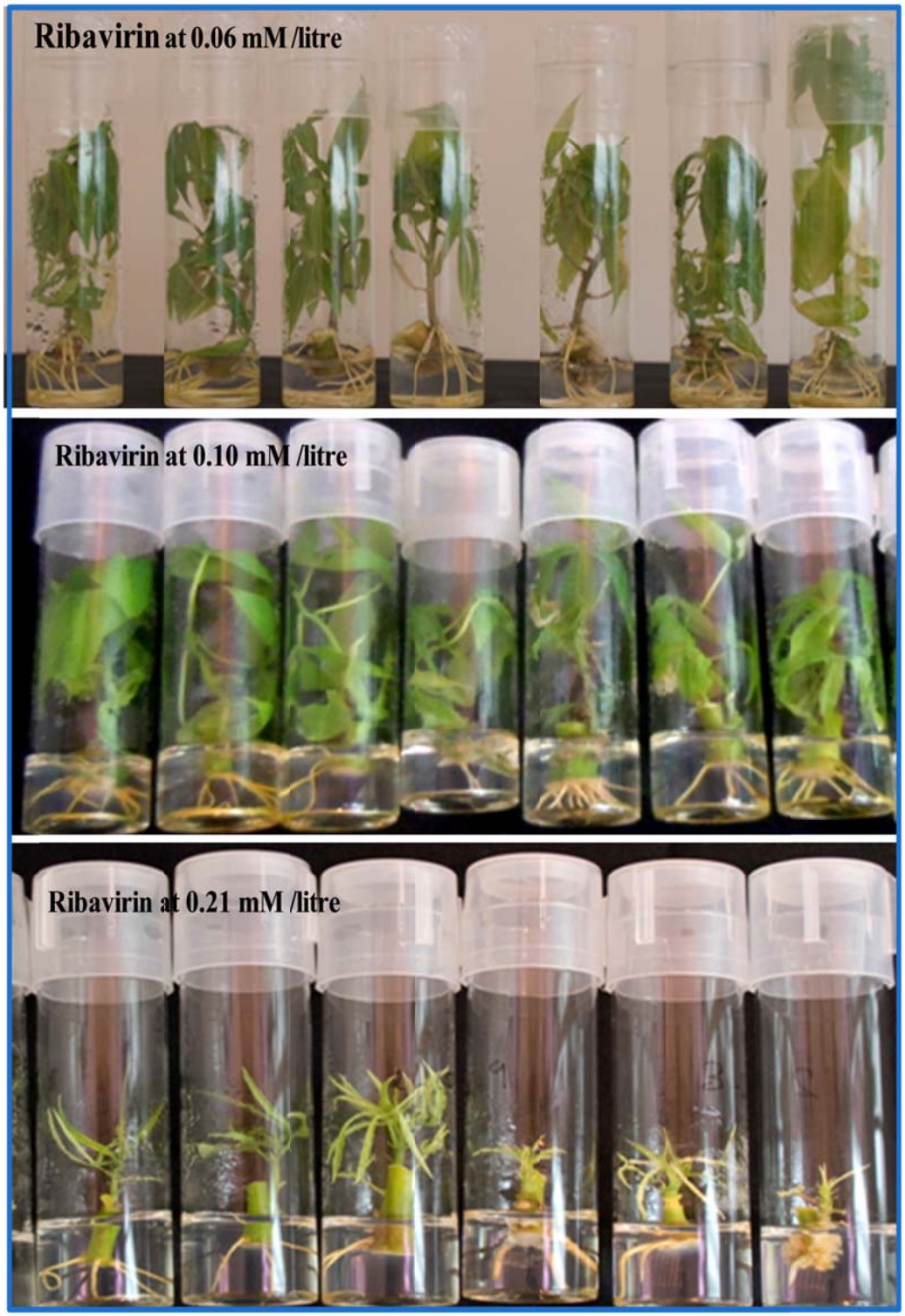



**Figure 7.3**: Regeneration of CBSD-infected node cuttings from cassava varieties by chemotherapy and reaction of the varieties at different ribavirin concentrations.


**Figure 7.4**: (a) Untreated control node cuttings, symptom free plants two months after transfer to the soil (b), UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-free plants of Kaleso (c) Kiroba and Albert (d).

Table 7.4: Effect of chemotherapy for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-elimination from infected cassava varieties.

| Chemo therapy | Number of plants regenerated or viruses-free by RT-PCR/Number tested[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UCBSV-[UG:Kab4-3:07] | | | | | | CBSV-[MZ:Nam1-1:07] | | | | | |
| | Kaleso | | Kiroba | | Albert | | Kaleso | | Kiroba | | Albert | |
| Ribavirin (mM/l)[a] | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f |
| 0.06 | 117/150 | 55/117 | 108/150 | 50/108 | 114/150 | 50/114 | 93/150 | 30/93 | 99/150 | 40/99 | 108/150 | 40/108 |
| 0.10 | 102/150 | 60/102 | 90/150 | 60/90 | 102/150 | 60/102 | 105/150 | 61/105 | 102/150 | 60/102 | 90/150 | 51/90 |
| 0.21 | 0/150 | nt | 0/150 | nt | 0/150 | nt | 0/150 | nt | 0/150 | nt | 0/150 | nt |
| Mean | 219/300 | 115/219 | 198/300 | 110/198 | 216/300 | 110/216 | 198/300 | 91/198 | 201/300 | 100/201 | 198/300 | 91/198 |

[a]150 nodes were inoculated into tissue culture media in each variety per isolate and antiviral chemical ribavirin (Rn) was added at different concentrations in millimolar per litre (mM/l). Results were recorded as number regenerated (No. reg) per inoculated, number virus free (V.f) per regenerated, nodes not tested (nt) due to contamination or dead. Plantlets were tested for the absence of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] by RT -PCR using CBSV specific primers (F3 and R3).

### 7.3.5 Simultaneous application of the therapies for *in vitro* regeneration of CBSD-affected cassava

Of the 30 nodes cultured in the tissue culture media supplemented with ribavirin at 0.10 mM/l (25 mg/l) and exposed to thermotherapy at 40 $^{o}$C, the greatest number of virus-free plants were found from UCBSV-[UG:Kab4-3:07]-infected plants compared to CBSV-[MZ:Nam1-1:07] (Table 7.5). Dual effects of thermo and chemotherapies, applied on *in vitro* cassava plants, were more efficient in eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] from the three cassava varieties than single treatment. However, regeneration of plantlets was low and this maybe due to the combined effects of the therapies resulting in leaf scorching caused by thermotherapy and phytotoxicity caused by ribavirin.

Table 7.5: Combined effect of the three therapies for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] elimination from infected cassava varieties.

| Combined therapies | TC+ TT + CT- UCBSV-[UG:Kab4-3:07]-elimination | | | | | | TC+ TT + CT -CBSV-[MZ:Nam1-1:07]-elimination | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Kaleso | | Kiroba | | Albert | | Kaleso | | Kiroba | | Albert | |
| Nodesa | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f | Reg. | V.f |
| No1 | 0/3 | nt | 0/3 | nt | 0/3 | nt | 0/3 | nt | 0/3 | nt | 0/3 | nt |
| No2 | 2/3 | 2/2 | 0/3 | nt | 1/3 | 1/1 | 1/3 | 1/1 | 0/3 | nt | 0/3 | nt |
| No3 | 2/3 | 2/2 | 1/3 | 1/1 | 2/3 | 2/2 | 0/3 | nt | 0/3 | nt | 2/3 | 2/2 |
| No4 | 3/3 | 3/3 | 2/3 | 2/2 | 3/3 | 1/3 | 2/3 | 2/2 | 1/3 | 1/1 | 2/3 | 1/2 |
| No5 | 0/3 | nt | 2/3 | 2/2 | 0/3 | nt | 0/3 | nt | 2/3 | 2/2 | 0/3 | nt |
| No6 | 1/3 | 1/1 | 3/3 | 2/3 | 2/3 | 2/2 | 1/3 | 1/1 | 3/3 | 2/3 | 2/3 | 2/2 |
| No7 | 0/3 | nt | 1/3 | 1/1 | 3/3 | 1/3 | 0/3 | nt | 1/3 | 1/1 | 1/3 | 1/1 |
| No8 | 3/3 | 3/3 | 1/3 | 1/1 | 3/3 | 2/3 | 2/3 | 1/2 | 1/3 | 1/1 | 2/3 | 2/2 |
| No9 | 2/3 | 2/3 | 2/3 | 2/2 | 2/3 | 0/2 | 1/3 | 1/1 | 2/3 | 2/2 | 2/3 | 1/2 |
| No10 | 3/3 | 2/3 | 3/3 | 2/3 | 1/3 | 0/1 | 2/3 | 2/2 | 3/3 | 3/3 | 3/3 | 1/3 |
| Mean | 16/30 | 15/16 | 15/30 | 13/15 | 17/30 | 9/17 | 9/30 | 8/9 | 13/30 | 12/13 | 14/30 | 10/14 |

[a]30 nodes from each variety per isolate were inoculated into tissue culture (TC) media supplemented with chemotherapy (CT, Ribavirin at 0.10 mM/l) and exposed to thermotherapy (TT, at 40 $^{o}$C). Results were recorded as number of plants regenerated (Reg.), and number virus-free (vf). Nodes not tested (nt) due to contamination or death.

### 7.3.6 Assessment of the efficacy of the therapies

The number of plantlets that regenerated from node cuttings varied between therapies and varieties (Table 7.6). For instance, 51% Kaleso, 69% Kiroba and 57% Albert plantlets regenerated from tissue cultured plants infected with UCBSV-[UG:Kab4-3:07]. A similar percentage of plants were regenerated from CBSV-[MZ:Nam1-1:07]-infected nodes (55% Kaleso, 62% Kiroba and 60% Albert). Simultaneous application of the three therapies resulted in decreased regeneration of plantlets in Kiroba for both viruses, while in Kaleso and Albert the decrease in plantlets regeneration was observed only in UCBSV-[UG:Kab4-3:07]. Simple tissue culturing of nodes resulted in the production of 49% and 18% of disease-free plants from Kaleso, 43% and 27% from Kiroba, and only 19% and 14% from Albert infected with UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07], respectively.

Elimination of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] by thermotherapy, was more efficient than chemotherapy (Table 7.6). Combining the three therapies together increased the elimination of both UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. The ET of tissue culture on the elimination of UCBSV-[UG:Kab4-3:07] was 25% for Kaleso, 10% for Kiroba, and 30% for Albert. The differences between the ET of thermotherapy and chemotherapy were not significantly different. Simultaneous application of the three therapies resulted in lowest ET (27%) from CBSV-[MZ:Nam1-1:07] and greatest (50%) from UCBSV-[UG:Kab4-3:07] on Kaleso (Table 7.6).

Table 7.6: Effect of therapies on regeneration of cassava plants for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-elimination.

| Tissue culture | % plantlets regenerated | | % virus elimination | | ET | |
|---|---|---|---|---|---|---|
| (TC) | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Variety | % | % | % | % | % | % |
| Kaleso | 51 | 55 | 49 | 18 | 25 | 10 |
| Kiroba | 69 | 62 | 43 | 27 | 30 | 17 |
| Albert | 57 | 60 | 19 | 14 | 11 | 9 |

| Thermotherapy | % plantlets regenerated | | % virus elimination | | ET | |
|---|---|---|---|---|---|---|
| (TT) | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Variety | % | % | % | % | % | % |
| Kaleso | 53 | 53 | 65 | 62 | 34 | 33 |
| Kiroba | 58 | 62 | 84 | 73 | 49 | 45 |
| Albert | 53 | 57 | 51 | 47 | 27 | 27 |

| | % plantlets regenerated | | % virus elimination | | ET | |
|---|---|---|---|---|---|---|
| Chemotherapy | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Variety | % | % | % | % | % | % |
| Kaleso | 73 | 66 | 53 | 46 | 38 | 30 |
| Kiroba | 66 | 67 | 56 | 50 | 37 | 34 |
| Albert | 72 | 66 | 51 | 46 | 37 | 30 |

| | % plantlets regenerated | | % virus elimination | | ET | |
|---|---|---|---|---|---|---|
| TC + CT + TT | UCBSV | CBSV | UCBSV | CBSV | UCBSV | CBSV |
| Variety | % | % | % | % | % | % |
| Kaleso | 53 | 30 | 94 | 89 | 50 | 27 |
| Kiroba | 50 | 43 | 87 | 92 | 44 | 43 |
| Albert | 66 | 47 | 53 | 71 | 35 | 33 |

Results are given in percentages, efficiency of therapy (ET) was determined as follows: % ET = % plant regenerated x % UCBSV-[UG:Kab4-3:07] or CBSV-[MZ:Nam1-1:07]-free plants / 100.

### 7.4 Discussion

Trials on the production of virus-free plants were carried out from node cuttings of CBSD-affected cassava plants of three different cassava varieties, using tissue culture, thermotherapy and chemotherapy. A comparison was made between node cuttings from positions 1-10 used as starting explants and the therapy efficiencies on CBSD-elimination. Node cuttings have been commonly used for plant propagation as well as for virus elimination. The death of nodes from positions 1-5 was greater compared to 6-10 in all treatments applied, including the control treatments. This is likely because the nodes from the top of the plants are tender and fragile while contamination with fungi and bacteria was more on the nodes from positions 6-10 which is likely to be the result of high concentrations of bacteria and fungi on lower parts of plants, due to concentration of photosynthates in phloem sap as they moved downward to the roots (Gibson and Otim-Nape, 1997).

The size of the node cuttings was important for initial growth, particularly in chemotherapy or thermotherapy. When node sizes of ~0.4 mm long were used a low number of plants were regenerated suggesting that the node size may be too small for plant growth. Large size nodes (0.6-0.8 mm) excised from yam had significantly better survival compared to small size nodes (0.3–0.5 mm) although it varied with the cultivar (Malaurie *et al*., 1998). The greatest virus elimination rate in yam was obtained with explants of 0.2-0.3 mm long, though the plant regeneration rate was decreased (Zapata *et al*., 1995). The effect of node size for regeneration and virus elimination in cassava was earlier reported by Kartha and Gamborg (1975) in which 135 of 150 plantlets were regenerated with 60% virus elimination when explants were excised at 0.4 mm, but increasing the node size to 0.5 mm and 0.8 mm resulted in all the plants regenerated, but exhibiting virus symptoms. Thus, the node sizes used in this study are efficient for virus elimination, but the plantlet's development may have been compromised.

Thermo and chemotherapies were compared for the regeneration of plants as well as for virus elimination. Three cassava varieties subjected to various therapies adapted differently in tissue culture. A comparatively greater number

of nodes developed from Kiroba and Albert than from Kaleso. Interestingly, plantlets from tissue culture alone and the ones treated with chemotherapy developed at a slower rate than those exposed to temperature regimes of 30 and 35 $^{o}$C. Node cuttings from cassava treated by thermotherapy at 35 $^{o}$C have also been reported to sprout quicker and develop into plantlets faster than the untreated ones (Kartha and Gamborg, 1975). Similar results were obtained in yams (Mantell *et al*., 1980; Chandler and Haque, 1984) and potatoes (Salazar and Fernandez, 1988), although the effect of heat on plantlet development is still unknown. Differences in the rate of virus elimination by thermotherapy were greater than other therapies in the three varieties. Like other therapies, thermotherapy was more efficient in eliminating mild UCBSV-[UG:Kab4-3:07] than the severe CBSV-[MZ:Nam1-1:07]. This is consistent with the elimination of potato viruses, in which thermotherapy was found to be more efficient in eliminating mild potato virus X (PVX) than severe potato virus S (PVS) (Stace-Smith and Mellor, 1968). Thus, the inactivation of virus with heat depended on the temperature regime used and the virus isolate as well as the host plant.

In this study greatest number of plantlets was regenerated at 35 $^{o}$C compared to other treatments. Similarly, a large number of plantlets were virus-free at 40 $^{o}$C when tested by RT-PCR, suggesting that the virus was inhibited by high temperature and new shoots produced during the thermotherapy could be virus-free (Kassanis, 1957). It was earlier speculated that under high temperature, the union of the protein sub-units (capsid) that protect the nucleic acid of the virus becomes weaker and temporal fissures appear, allowing attack by nucleases (Allam, 2000). The high rate of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-elimination at high temperature could be attributed to the possibility that increased temperatures destroy essential chemical processes in the virus life cycle. The percentage of virus-free plants obtained from thermotherapy in this study (47% from CBSV-infected Albert to 84% from UCBSV-infected Kiroba) is high compared to that of Wasswa *et al*. (2010) (49%) at 40 $^{o}$C. Walkey (1976) further demonstrated that *Cucumber mosaic virus* (CMV) did not multiply at 30 $^{o}$C in *N. rustica*, the virus was completely inactivated at 32 $^{o}$C and the virus was eliminated after 30 days.

The highest3 temperature used in this study (45 $^{o}$C) resulted in death of all the plantlets of the three cassava varieties, indicating the temperature threshold at which cassava nodes cannot survive.

The use of ribavirin at different concentrations did not positively influence development of plantlets which was contrary to the results obtained by Nascimento *et al.* (2003) who observed increased potato development in the media supplemented with antiviral chemicals. A threshold was reached in this study at 0.21 mM/l ribavirin concentration, where all the node cuttings from the three cassava varieties did not survive (Figure 7.3; Table 7.4). Best plant regeneration was registered when ribavirin was used at 0.10 mM/l, but plant development was slow even at this concentration compared to the control plants and other therapies. Ribavirin has also been shown to slow the regeneration of potatoes (Klein and Livingston, 1982; Slack *et al*., 1987). These observations confirmed that ribavirin is toxic for the *in vitro* development of cassava and other plants. It was noted that developing virus-free plants by chemotherapy (Klein and Livingston, 1982) will take longer than thermotherapy (Stace-Smith and Mellor, 1968) or tissue culture alone.

Combined effects of the thermo and chemotherapies on *in vitro* cassava were highly efficient in eliminating UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]. Treatments that included addition of ribavirin at 0.10 mM/l into the tissue culture media and exposure to 40 $^{o}$C resulted in increased virus-elimination compared to single treatments. Similar results were obtained from PVY elimination in potato by Nascimento *et al.* (2003). The rates obtained in this study were greater than those obtained by Dunbar *et al.* (1993), who eliminated *Peanut mottle virus* (PeMoV) in 24% of plants.

The ET for UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07]-elimination varied between the therapies and cassava varieties used. Thermotherapy was most efficient for eliminating both viruses from Kaleso and Kiroba when compared to Albert, in which chemotherapy alone was more efficient for eliminating UCBSV than the simultaneous application of the three therapies. Chemotherapy had the main disadvantage of using a chemical

Ribavirin which is toxic to human and plant tissue and they were also expensive (Klein and Livingston, 1982; Ng *et al*., 1992; James *et al*., 1997). Thermotherapy may well be an efficient method if the period of heat exposure is extended from 21 days used in this study to 30 days or more. Thermotherapy is therefore considered to be the preferred method due to high rates of virus elimination and high plant regeneration.

### 7.4.1 Conclusions

The main conclusions arising from Chapter 7 are:

1. The regeneration of *in vitro* cassava plantlets was greater from node cuttings numbered 6-10 from the top than nodes from position 1-5 while virus elimination was greater from the top part of the plants than from the bottom.

2. Both UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] can be eliminated from cassava using *in vitro* tissue culture, thermotherapy, chemotherapy or simultaneous application of the therapies but at varied efficiencies depending on the variety.

3. Cassava varieties subjected to various therapies adapted differently in tissue culture. A comparatively greater number of nodes developed from Kiroba and Albert than from Kaleso.

4. Thermotherapy was most efficient for eliminating both viruses from Kaleso and Kiroba when compared to Albert, in which chemotherapy alone was more efficient than the simultaneous application of the three therapies.

## CHAPTER 8: General Discussions and Conclusions

The current study established vital concepts underlying the interactions of UCBSV and CBSV with the host cassava in the CBSD-pathosystem. Studies on symptom severity on cassava and herbaceous host plants has identified the presence of severe and milder forms of CBSVs. CBSV-[MZ:Nam1-1:07] from Mozambique and CBSV-[TZ:Nal3-1:07] from Tanzania expressed severe symptoms on cassava, while UCBSV-[UG:Kab4-3:07] from Uganda, and to some extent UCBSV-[TZ:Kib10-2:03] from Tanzania expressed relatively milder symptoms. This observation was consistent on herbaceous host plants, *N. clevelandii* and *N. benthamiana*, as plants infected with CBSV-[MZ:Nam1-1:07] and CBSV-[TZ:Nal3-1:07] were severely stunted and subsequently wilted while those infected with UCBSV-[TZ:Kib10-2:03] and UCBSV-[UG:Kab4-3:07] developed various patterns of mild chlorosis, but not necrosis and death. The severity of the viruses was because of their ability to increase in titre in infected plants, which was confirmed by serial dilution of viral cDNA which indicated that severe viruses were detectable at $10^{-5}$ while the milder isolates were not detected below $10^{-3}$ dilutions or less. Our study further agreed with the study conducted by Moreno *et al*. (2011) in which the CBSD symptom severity correlated with high virus titre.

When plants were clonally propagated to determine virus severity and the effect of CBSD on sprouting of infected cuttings, maximum number of cuttings were sprouted from plants infected by the milder UCBSV isolates (92%) and a relatively smaller number from plants infected by the severe CBSV isolate (58%). This may be due to the hyper virulent nature of the severe CBSV isolates (Nichols, 1950), which killed plants in fields. Spread of these viruses into areas of high whitefly populations and the possibility of mixed infections of UCBSV and CBSV are likely to cause even more severe damage to cassava production than yet encountered. One such area that needs further study is therefore the possibility of synergism between UCBSV and CBSV isolates.

Early workers on CBSD also described variation in leaf symptoms (Nichols, 1950; Storey, 1939) and this was attributed to the inherent response to

infection of the respective varieties (Jennings, 1960b). Information on severity of symptoms induced, host range and mode of transmission are vital for virus classification, especially when differentiating between viruses (Shukla *et al*., 1988). Moreover, information on virus host range and means by which it is transmitted, as well as the different isolates involved in disease development are important requirements for developing appropriate virus control methods (Mathew, 1991).

Successful transmission of the viruses was achieved by sap inoculation of herbaceous host plants from cassava and also from herbaceous to herbaceous host plants, but the rate of sap transmission (17% and 23%) from cassava to cassava was low. These results are in agreement with Lister (1959) as transmission and spread of UCBSV and CBSV was considered to be mainly through vectors and perpetuating infected cuttings (Hillocks *et al.,* 2001; Maruthi *et al.*, 2005). In addition to confirming UCBSV and CBSV transmission by methods including vectors, perpetuation through use of infected cuttings, sap and graft inoculation, it was established in this study for the first time that CBSVs are not transmitted through contaminated cutting tools and harvesting of cassava leaves for vegetable consumption as is being practiced in some countries or animal feeding. This is contrary to many other plant viruses and virus like particles such as PVX and potato tuber viroid, which were found to be transmitted by contaminated tools (Manzer and Merriam, 1961). These results suggest that leaf picking or the use of contaminated tools are not responsible for the recent upsurges in CBSD incidences and control strategies should emphasise the use of clean planting material.

Graft-transmission of CBSV gave 100% infection in susceptible varieties making it the most reliable means of virus transmission in experiments. The high whitefly transmission rates observed in this study (57%) compared to low rates obtained by Maruthi *et al*. (2005) and Mware *et al.* (2009), however is comparable to transmission rates of other ipomoviruses (*Cucumber vein yellowing virus*, CVYV, 55%) by whitefly (Mansour and Al-Musa, 1993); this could be due to some technical updates in the protocol followed such as

allowing the whiteflies to acquire the virus freely on a diseased plant and using a set AAP and IAP of 24 h. All six UCBSV and CBSV isolates used in this study were transmitted to healthy cassava plants by graft-inoculation and resulted in virus infection without difficulty. Therefore, to improve our understanding of the mechanisms of CBSD resistance in cassava, graft-inoculation was preferred. Other researchers and plant breeders can also conveniently use the method to inoculate cassava plants with the target virus without the need for using whitefly transmissions. Graft inoculation is quick to determine when an inoculation is successful through the survival of the graft. However, the virus challenge in the graft-inoculated plant is greater than challenge by whitefly inoculations and this may result in varieties with usable field resistance being discarded.

The differences identified in the levels of resistance were shown to be due to a combination of the interactions between the virulence of viruses and the inherent resistance mechanisms of the plant. Legg (1994) and Solomon-Blackburn and Baker (2001) described mechanisms to be considered while selecting varieties for resistance. Firstly, virus multiplication at the early stages of infection is delayed or prevented. Secondly, is the hypersensitive reaction (HR), which is the ability of the variety to prevent spread of infection to other parts of the plant beyond the immediate site of invasion (Cooper, 2001). The third mechanism is the resistance to vectors. Another mechanism is resistance to virus accumulation, where plants are infected and the virus spreads in the plant, but virus titre is very low. In this study the virus moved quicker in Albert which is a known susceptible variety, than in Kiroba (tolerant) and Kaleso (field-resistant). Both UCBSV and CBSV first spread down to the root and then to the rest of the plant, which was similar to the pattern of spread of ACMV (Gibson and Otim-Nape 1997). Regarding resistance to vectors, *B. tabaci* fecundity and survival studies on Kaleso, Kiroba and Albert demonstrated the absence of significant differences between the ability of cassava varieties to support *B. tabaci* development. This was in agreement with the observations of Maruthi *et al*. (2001) and Hahn *et al*. (1980) who noted no differences in *B. tabaci* survival on CMD-resistant, tolerant and susceptible cassava varieties and thus concluded that resistance to

the vector was unlikely to be found in cassava. In the absence of differences between the varieties for *B. tabaci* development, our results lead to the following conclusions: first, resistance to UCBSV and CBSV in cassava is not because they are unattractive to *B. tabac*.

The virus titre in the susceptible Albert was high compared to Kiroba and Kaleso. The three cassava varieties used in this study expressed different CBSD symptom severities that matched with virus titre. CBSV-[MZ:Nam1-1:07] titre in Albert was associated with severe symptoms. Albert infected with UCBSV-[UG:Kab4-3:07] expressed milder symptoms and had low virus titre than the infection of the same varieties with CBSV-[MZ:Nam1-1:07]. The milder UCBSV-[UG:Kab4-3:07] was also not associated with root necrosis in varieties Kiroba and Kaleso which is in agreement with the findings of Hillocks *et al*. (1996) that some cassava varieties expressed foliar symptoms with or without root necrosis.

Reversion is another resistance mechanism earlier recognised in CMD-resistant cassava varieties in East Africa (Storey and Nichols, 1938; Jennings, 1957; Rossel *et al*., 1992; Thresh *et al*., 1994; Fargette *et al*., 1996; Gibson and Otim-Nape, 1997) and seemed to work on both local and improved cassava varieties (Fondong *et al.,* 2000). After infection with viruses, plants employ RNA silencing mechanism against all foreign genes entering the plant (Vaucheret, 2001; Voinnet, 2001). However, many viruses, in turn, employ virus-encoded proteins which suppress RNA silencing allowing them to successfully infect their host (Kasschau and Carrington, 1998; Voinnet *et al*., 2000; Ahlquist, 2002; Moissiard and Voinnet 2004). In turn, however, plants also evolved an even greater level of host resistance that restrain virus-encoded RNA silencing suppression (Li *et al*., 1999) which is manifested through possibilities of diseased plants to revert from virus infection. Reversion also seems to work on the RNA silencing mechanism (Ratcliff *et al*., 1999; Kreuze *et al*., 2002) but, severely CBSD affected plants do not revert; the mechanism seems to be commonly deployed for more tolerant varieties and reversion was observed especially from milder UCBSV and more frequently in more resistant varieties for CBSD for the first time in this study.

It was previously suggested by Hillocks and Jennings (2003) that reversion in CBSD-infected cassava is due to localised distribution of the virus. Reversion was greatest in cuttings taken from the middle and upper portion of the stem than from the bottom. Likewise, Gibson and Otim-Nape (1997) observed high reversion from middle and upper portions of CMD-infected cassava compared to least number from lower part of the stems. Although the greatest rate of reversion occurred in shorter cuttings of 10 cm long, short cuttings were less viable and grew weakly (Gibson and Otim-Nape, 1997), which could predispose them to CBSVs re-infection, attack by pest and other pathogens and this, may lead to poor yield. Cuttings of intermediate length of 15 cm therefore will be suitable to achieve an optimum rate of reversion and acceptable plants.

Besides the natural potential of some varieties to revert from UCBSV and CBSV infection, eliminating UCBSV and CBSV from infected cassava (Chapter 7) was investigated using thermo and chemotherapies, or simple nodal culture. These eliminated both UCBSV and CBSV from infected cassava. Tissue culturing alone resulted in virus elimination (up to 30%) of plants and regeneration of relatively high number of virus-free plantlets in a short period, suggesting a high potential of the *in vitro* methods for regenerating virus-free cassava from CBSV-infected plants. Virus elimination from these methods can be useful especially for the elite but susceptible varieties infected with severe isolates from which they do not easily revert. During heat treatment, there are probably unsuitable conditions for virus movement and replication, thus the node cuttings elongate faster than the rate at which the virus moves to the top. High metabolic activity observed in the callus was well reported to interfere with virus replication due to competition for resources (Valentine *et al.,* 2002). Virus elimination from potato was achieved by the combination of thermotherapy and the addition of ribavirin to the tissue culture media (Dodds *et al.,* 1989; Griffiths *et al.,* 1990; Fletcher *et al*., 1998). The combined effects of thermo and chemotherapies in this study on cassava were highly efficient in eliminating both UCBSV-[UG:Kab4-3:07] (53 to 94%) and CBSV-[MZ:Nam1-1:07] (71 to 92%). A significant drawback with *in vitro* techniques, however, is that cassava varieties differed greatly

when planted in the tissue culture media therefore the protocol should be optimised for each variety (Kaiser and Teemba, 1989).

In conclusion, the current study adopted a number of approaches to study the relationship between CBSV-infection and symptoms expression. The severity of the disease depends on the tolerance level of the variety, virus isolate and the duration of infection. It was further demonstrated that there are differences in the susceptibility between the tested cassava varieties which are not due to differences in their ability to attract and support *B. tabaci*. Similarly, there were differences in pathogenicity between the test virus isolates with two viruses having been identified, one of which is associated with the Uganda epidemic. Virus isolate from Uganda was less pathogenic than the Mozambique isolate. Protocols were established for the efficient graft-inoculation with 100% infection of susceptible cassava varieties. Differences identified in the levels of susceptibility following graft-inoculation, are related to different rates of virus movement and multiplication after initial infection. UCBSV and CBSV first spread down to the root, then to the rest of the plant. Virus titre results can indicate varieties with high reversion potential and such varieties can then be used to breed for resistance to viruses.

Reversion was shown to occur with CBSD and is frequent in resistant varieties. Therefore, CBSV-free plants can be generated from diseased plants. Heat and chemical methods can be used to eradicate both UCBSV and CBSV from infected plants. RT-PCR results indicated that tissue culturing alone had a positive effect in removing the virus from 9 to 30% of the plants. The ET for thermotherapy and chemotherapy ranged from 27 to 49%, and 30 to 38% respectively, while the ET of the combined therapies ranged from 33 to 50%. Consequently, chemotherapy is considered as a least effective method due to its low efficiency as well as the toxic nature of ribavirin. Further work is needed on; (1) the relationship between symptom severity and virus titres in different cassava varieties, (2) the nature of interactions between UCBSV and CBSV and UCBSVs in dual infected cassava plants, (3) rates of UCBSV transmission by whitefly on different cassava varieties, (4) other mechanisms of UCBSV and CBSV transmissions and (5) influence of different cassava

varieties, CBSD isolates and environment on the mechanism of resistance to CBSD. Research gaps cited above open up opportunities for the academic virologists, plant breeders, molecular biologists and/or researchers. The findings from this study have contributed significantly to improve our understanding of the role of host cassava, virus and the vector whitefly in causing CBSD. In view of existing damage and threats posed by CBSD, it is essential to identify and then implement effective management strategies for the disease. Recognizing the existence of two forms of the virus and important differences between them will greatly aid the development of effective strategies. Management tactics for CBSD should seek for the high level of effective resistance. This highlights the importance of developing host plant resistance to CBSD that is of comparable robustness to that currently available for CMD (Legg *et al*., 2011). This may likely be achieved if both conventional and transgenic breeding approaches are explored. Equally important, is the recognition of reversion to CBSD. This will help minimize the perpetuation of CBSV and UCBSV through infected cuttings. The rate of transmissions of CBSV obtained in this study suggests that whitefly management will not only provide a solution to current CBSD pandemics, but in addition, will significantly reduce the likelihood for the emergence of new epidemics caused by variant isolates.

## REFERENCES


Abarshi, M.M., Mohammed, I.U., Wasswa, P., Hillocks, R.J. Holt, J. Legg, J.P., Seal, S.E. and Maruthi, M.N. (2010). Optimization of diagnostic RT-PCR protocols and sampling procedures for the reliable and cost-effective detection of Cassava brown streak virus. *Journal of Virological Methods,* **163**: 353-359.

Abarshi, M.M., Mohammed, I.U. Jeremiah, S.C. Legg, J.P. Lava Kumar, P. Hillocks, R.J. Maruthi M.N. (2012). Multiplex RT-PCR assays for the simultaneous detection of both RNA and DNA viruses infecting cassava and the common occurrence of mixed infections by two cassava brown streak viruses in East Africa. *Journal of Virological Methods,* **18**: 176-179.

Abouzid, A.M., Freitas-Astua, J., Purcifull, D.E., Polston, J.E., Beckham,K.A., Crawford, W.E., Petersen, M.A., Peyser, B., Patte, C. and Hiebert, E. (2002). Serological studies using polyclonal antisera prepared against the viral coat protein of four begomoviruses expressed in *Escherichia coli*. *Plant Disease,* **86**: 1109-1114.

Adams, M.J, Antoniw, J.F, and Fauquet, C.M. (2005). Molecular criteria for genus and species discrimination within the family *Potyviridae*. *Archives of Virology*, **150**: 459-479.

Adams, M. (2008). Descriptions of plant viruses. Cleavage sites in the family *Potyviridae***.**http://www.dpvweb.net/potycleavage/index.html. Accessed on 12/08/2011.

Agboola, S.A. (1968). The introduction and spread of cassava in Western Nigeria. *Economic and Social Studies,* **10**: 369-385.

Agwu, A.E and Anyaeche, C.L (2007) Adoption of Improved Cassava Varieties in six rural Communities of Anambra State, Nigeria. *African Journal of Biotechnology*, **6**: 089-098.

.Ahmad, M., Arif, M.I. and Denholm, I. (2003). High resistance of field population of cotton aphid (*A. gossypii*) to pyrethroid insecticides in Pakistan. *Journal of Entomology*, **96**: 875-878.

Ahlquist, P. (2002). RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science*, **296**: 1270-1273.

Akhtar, M., M. Ashraf and M.E. Akhtar (2003).Sugarcane yield gap analysis: Future options for Pakistan. *Science Technology and Development*, **1**: 38-48.

Akinbade, S.A., Hanna, R., Nguenkam, A., Njukwe, E., Fotso, A., Doumtsop, A., Ngeve, J., Tenku, S.T.N., Lava Kumar, P. (2010). First report of the East African cassava mosaic virus-Uganda (EACMV-UG) infecting cassava (Manihot esculenta) in Cameroon. *New Disease Report*, **21**: 22.

Alicai, T., Omongo, C.A., Maruthi, M.N., Hillocks, R.J., Baguma, Y., Kawuki, R., Bua, A., Otim-Nape, G.W. and Colvin, J. (2007). Re-emergence of cassava brown streak disease in Uganda. *Plant Disease*, **91**:24-29.

Alabi, O.J., Kumar, P.L., Naidu, R.A. (2008). Multiplex PCR for the detection of African cassava mosaic virus and East African cassava mosaic Cameroon virus in cassava. *Journal of Virology Methods*, **154**, 111-120.

Allam, E.K. (2000). Eradication of *Banana bunchy top virus* and *Banana mosaic virus* from diseased banana plants. *Annals of Agricultural Science,* **45**: 33-48.

Allem, A.C. (2002). The origins and taxonomy of cassava. In: *Cassava: biology, production and utilization.* Cambridge University Press. CAB International, Wallingford, UK. Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). Pp 1-16.

Alves, A.A.C. (2002). Cassava botany and physiology, In: In: *Cassava: biology, production and utilization.* Cambridge University Press. CAB

International, Wallingford, UK. Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). Pp 67-89.

Ammar, E.D. Järlfors, U., Pirone, T.P. (1994). Association of potyvirus helper component protein with virions and the cuticle lining the maxillary food canal and foregut of an aphid vector. *Phytopathology*, **84**: 1054-60.

Andrejeva, J., Puurand, Ü., Merits, A., Rabenstein, F., Järvekülg, L. and Valkonen, J.P.T. (1999). Potyvirus HC-Pro and CP proteins have coordinated functions in virus-host interactions and the same CP motif affects virus transmission and accumulation. *Journal of General Virology,* **80**: 1133-1139.

Balagne, M. (1985). Le microbouturage in vitro de l'igname cousse couche Dioscorea trifida en vue de son application pour la guérison des variétés atteintes de viroses. Thèse de 3ème cycle, USTL, Montpellier, 144p.

Bamikole, O.T. (2007). Production of fuel ethanol from cassava in Nigera: obstacles and benefits. In: *10th Triennial Symposium jointly organized by the ISTRC-AB and Mozambique Agrarian Research Institute* (IIAM). Joaquim Chissano International Conference Centre, Maputo, Mozambique 8-12 October 2007. Mahungu, M.N. and Mkumbira, J. (eds.) 10p.

Barratt, C. B., Marenya, P.P., McPeak, J., Minten, B., Murithi, F., Oluoch-Kosura, W. (2006). Welfare dynamics in rural Kenya and Madagascar. *Journal of Development Studies*, **42**: 248-277.

Baulcombe, D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *The Plant Cell,* **8**: 1833-1844.

Bellotti, A.C. (2002). Arthropod pests, In: Cassava: Biology, Production and Utilization. Cambridge University Press. CAB International, Wallingford, UK Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds.). Pp 209-235.

Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, **409**:295-296.

Berrie, L. C., Palmer, K. E., Rybicki, E. P. and Rey, M. E. C. (1998). Molecular characterisation of a distinct South African cassava infecting geminivirus. *Archives of Virology,* **143**: 2253-2260.

Bird, J. (1957). A whitefly transmitted mosaic of Jastroph gossypifolia. Technical Paper. *Agricultural Experiment Station Puerto Rico*, **22**: 1-35.

Bird, J. and Maramorosch, K. (1978). Viruses and virus diseases associated with whiteflies. *Advances in Virus Research*, **22**: 55-110.

Bock, K.R, Guthrie, E.J. and Meredith, G. (1978) Distribution, host range, properties and purification of cassava latent virus, a geminivirus. *Annal of Applied Biology*, **90**: 361-367.

Bock, K.R. (1982). Geminivirus diseases in tropical crops. *Plant Disease*, **66**: 266–270.

Bock, K.R. (1983). Epidemiology of cassava mosaic in Kenya. In: Plant virus disease epidemiology. Blackwell, Oxford. Plumb, R.T. and Thresh, J.M. (eds.) Pp 337- 347.

Bock, K.R., Woods, R.D. (1983). The etiology of African cassava mosaic disease. *Plant Disease*, **67**:994-995.

Bock, K.R. (1994). Studies on cassava brown streak virus disease in Kenya. *Tropical Science Journal*, **34**:134-145.

Boevink, P. and Oparka, K.J. (2005). Virus–host interactions during movement processes. *Plant Physiology*, **138**:1815-1821.

Boissot, N., Urbino, C., Dintinger, J., Pavis, C. (2008). Vector and graft inoculations of Potato yellow mosaic virus reveals recessive resistance in Solanu pimpinellifolium. *Annalsof Applied Biology*, **152**: 263-269.

Bokanga A. and Otoo, E., (1991). Cassava based food, How safe are they? In K.A. Taiwo, Utilization Potentials of cassava in Nigeria: The Domestic and Industrial products. *Food Reviews International*, **22**:29-42.

Bokanga, M. and Otoo, E. (1994). Cassava based food: how safe are they In: Proceedings of the 9th Symposium of the International Society for Tropical Root Crops, held at Accra, Ghana 20-26 October 1991. Ofori, F. and Hahn, S.K. (eds.), 225p.

Bokanga, M., Ekanayake, I.J. and Dixon, A.G.O. (1994). Genotype-Environment interactions for cyanogenic potential in cassava. In: *Proceedings of the International Workshop on Cassava Safety,* held at Ibadan, Nigeria, March 1 – 4, 1994. Bokanga, M., Essers, A.J.A., Poulter, N., Rosling, H. and Tewe, O. (eds.). 132p.

Bokanga, M. (2007). Root crops biofuels and food security in Africa. In: *10th Triennial Symposium jointly organized by the ISTRC-AB and Mozambique Agrarian Research Institute* (IIAM). Joaquim Chissano International Conference Centre, Maputo, Mozambique 8-12 October 2007. Mahungu, M.N. and Mkumbira, J (eds.) 7p.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana. EMBO Journal,* **17**: 6739-6746.

Broadbent P, Gollnow B.I., Gillings M.R. and Bevington K.B., (1968). Root Grafting and Mechanical Transmission of Citrus Exocortis Viroid Within a Citrus Budwood Multiplication Block. http://www. ivia. es/iocv/ archivos /proceedings X/10th197_ 203 .pdf. accessed on 20/02/2011.

Brown, J.K. (1990). An update on the whitefly-transmitted geminiviruses in the Americas and the Caribbean Basin. *FAO Plant Protection Bulletin,* **39**: 5-23.

Brown, J.K., Frohlich, D.R. and Rosell, R.C. (1995). The sweetpotato or silverleaf whiteflies: biotypes of Bemisia tabaci or a species complex? *Annual Review of Entomology*, **40**: 511–534.

Brunt, A.A. (1996). Cassava brown streak – carlavirus? cassava brown streak potyvirus? In: Plant Viruses Online journal. http:// biology. anu. edu. Au /Groups /ME. Accessesd: 20/10/2008.Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (eds.). Pp 56-63.

Burban, C., Fishpool, L.D.C, Fauquet, C., Fargette, D. and Thouvenel, J.C. (1992). Host-associated biotypes within West African populations of the whitefly Bemisia tabaci (Genn.), Homoptera: Aleyrodidae. *Applied Journal of Entomology*, **113**: 416-423.

Bustin, S.A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009). The MIQE Guidelines:Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry,* **55:** 611-622.

CABRI (1998). Grafting of virus infected plant material to healthy host plants. Laboratory procedures for plant cell viruses. The CABRI Consortium 1999-2003.http:// www. cabri. org/ guidelines/ plant_virus/ pcv202ap3. html. accessed on 23/04/2011.

Calvert, L.A. and Thresh, J.M. (2002). The viruses and virus diseases of cassava. In: *Cassava: Biology, Production and Utilization*. Cambridge University Press. CAB International, Wallingford, UK Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). Pp 238-242.

Carrington, J.C. and Whitham, S.A. (1998). Viral invasion and host defense: strategies and counter-strategies. Institute of Biological Chemistry, Washington State University press. Pullman, USA. 336p.

Carrington, J.C. and Ambros, V. (2003). Role of microRNAs in plant and animal development. *Science,* **301**: 336-338.

Carter, S.E., Fresno, L.O., Jones, P.G. and Fairbairn, J.N. (1992). An atlas of cassava in Africa: Historical, Agroecological and Demographic aspects of crop distribution. CIAT, Cali Colombia. Pp 2-9.

Cassels, A.C. and Long, R.D. (1982). The elimination of potato viruses X,Y,S and M in meristem and explants cultures of potato in the presence of virazole. *Potato Research*, **25**: 165-173.

Chabwine, J.N., Masheka, C., Balol'ebwami, Z., Maheshe, B., Balegamire, S., Rutega, B., Lola, M., Mutendela, K., Bonnet, M.-J., Shangalume, O. and Balegamire, J.M Nemery, B. (2011). Appearance of konzo in South-Kivu, a wartorn area in the Democratic Republic of Congo. *Food Chemical Toxicology*, **49**: 644–649.

Chandler, F.L. and Haque, S.Q. (1984). The use of tissue culture in the production of improved yam and sweet potato planting material. In: Micropropagation of selected Root crops, palm, citrus and ornamental species. FAO plant production and protection paper, N0. 59 Rome.

Chávez, A.L., Sánchez, T., Jaramillo, G., Bedoya, J.M., Echeverry, J., Bolaños, A., Ceballos, H., and Iglesias, C.A. (2005). Variation of quality traits in cassava roots evaluated in landraces and improved clones. *Euphytica*, **143**: 125-133.

Chen, J. and Adams, M.J. (2001). A universal PCR primer to detect members of the Potyviridae and its use to examine the taxonomic status of several members of the family. *Archieves of Virology*, **146**: 757-766.

Cliff, J., Muquingue, H., Nhassico, D., Nzwalo, H., Bradbury, J.H. (2011). Konzo and continuing cyanide intoxication from cassava in Mozambique. Workshop on Toxico-nutritional neurodegenerations, September 2009, IPBO-Ugent, Belgium. *Food Chemical Toxicology*, **49**: 631-635.

Colinet, D., Kummert, J. and Lepoiver, P. (1996). Molecular evidence that the whitefly – transmitted sweet potato mild mottle virus belongs to a distinct genus of the Potyviridae. *Archives of Virology*, **141**:125-135.

Colinet, D., Kummert J., and Lepoivre P. (1998). The nucleotide sequence and genome organization of the whitefly transmitted sweetpotato mild mottle virus: a close relationship with members of the family Potyviridae. *Virological Research*, **53**: 187-196.

Covey, S.N., Al-Kaff, V.S., Lángara, A. and Turner, D.S. (1997). Plants combat infection by gene silencing. *Nature,* **385**: 781-782.

Cooke, R.D. and Coursey, D.G. (1981). Cassava: a major cyanide-containing food crop. In: *Cyanide in Biology.* Vennesland, B. Conn, E.E. and Knowles, C.J. (eds.). Academic Press, New York. Pp 94-113.

Cooper, B. (2001). Collateral gene expression changes induced by distinct plant viruses during the hypersensitive resistance reaction in Chenopodium amaranticolor. *The Plant Journal*, 26: 339-349.

Crawley, M.J. (2009). *Statistics an introduction using R*... Imperial College London UK: John Willey and Sons Ltd. 253P.

Dalmay, T., Horsefield, R., Braunstein, T.H. and Baulcombe, D.C. (2001). SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. *The EMBO Journal,* **20**: 2069-2077.

Deng, D., McGrath, P.F., Robinson, D.J. and Harrison, B.D. (1994). Detection and differentiation of whitefly-transmitted geminiviruses in plants and vector

insects by the PCR with degenerate primers. *Annals of Applied Biology,* **125**: 327-336.

Devries, S.C., Gerrie W.J. Martink, V., Ittersum, V. and Giller K. (2011). The production-ecological sustainability of cassava, sugarcane and sweet sorghum cultivation for bioethanol in Mozambique. *GCB Bioenergy,* **10**: 1757-1707.

Dixon, A.G.O., Ngeve, J.M. and Nukenine, E.N. (2002). Response of Cassava Geno-types to four Biotic Constraints in Three Agro-ecolgies of Nigeria. *African Crop Science Journal,* **10**: 12.

Dodds, J.H.; Lizarraga, R.; Griffiths, H.; Slack, S.A. (1989). Methods of virus eradication. In: *Planning coference on control of virus and virus-like diseases of potato and sweet potato*, Lima, 1989. Report 3,. Lima: International Potato Center, 228p.

Dunbar, K.B.; Pinnow, D.L.; Morris, J.B.; PIttman, R.N. (1993). Virus elimination from interspecific *Arachis* hybrids. *Plant Disease*, **77**: 517-520.

Ekanayake, I.J., Osiru, D.S.O. and Porto, M.C.M. (1997). Agronomy of cassava. IITA *Research Guide* 61. Training Program, IITA, Ibadan, Nigeria. First edition. 30p.

Elbashir, S.M., Leneckel, W. and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes and Development,* **15**:188-200.

Elia, A., Constantinou, C. and Clemens, M.J. (2008). Effects of protein phosphorylation on ubiquitination and stability of the translational inhibitor protein 4E-BP1. *Oncogene,* 27: 811–822.

Encina, C.L., Constantin, M. and Botella, J. (2001). An easy and reliable method for establishment and maintenance of leaf and root cell culture of *Arabidopsis*. *Plant Biology report.* **19**: 245-248.

Ernesto, M., Cardoso, A.P., Nicala, D., Mirione, E., Massaza, F., Cliff, J., Haque, M.R., Bradbury, J.H. (2002). Persistent konzo and cyanogen toxicity from cassava innorthern Mozambique. *Acta Tropica,* **82**: 357-362.

Ezeibekwe, I.O., Ezenwaka, C.L., Mbagwu, F.N. and Unamba C.I.N. (2009). Effect of combination of different level of auxin (NAA) and Cytokinin (BAP) on *in vitro* propagation of *Dioscorea rotundata* L (White yam). *Journal of Molecular Genetics*, **1**: 18-22.

Fargette, D., Fauquet, C. and Thouvenel, J.C. (1985). Field studies on the spread of African cassava mosaic. *Annals of Applied Biology,* **106**: 285- 294.

Fargette, D., Colon, L.T., Bouveau, D. and Fauquet, C.M. (1996). Components of resistance of cassava to African cassava mosaic virus. *European Journal of Plant Pathology,* **102**: 645-654.

Fauquet, C.M. and Fargette, D. (1990). African cassava mosaic virus: Aetiology, epidemiology and control. *Plant Disease,* **74**: 404-011.

FAOSTAT, (2009). Production Statistics. http//faostat.fao.org accessed 23/07/2010.

FAOSTAT, (2010). Production Statistics. http//faostat.fao.org accessed 03/04/2011.

Ferguson, G. (2009). Potential New Invasive Pests for Canadian Greenhouse Vegetable Crops. In: *Canadian Greenhouse* (C.G.C.) held on 7th October 2009. www.agric.wa.gov.au accessed on 23/10/11.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, SE and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans. Nature,* **391**:806-811.

Fletcher, P.J.; Fletcher, J.D.; Cross, R.J. (1998). Potato germplasm: in vitro storage and virus reduction. *New Zeland Journal of Crop and Horticultural Science*, **26**: 249-252.

Fondong, V.N., Thresh, J.M. and Fauquet, C. (2000). Field experiments in Cameroon on cassava mosaic virus disease and the reversion phenomenon in susceptible and resistant cassava cultivars. *International Journal of Pest Management*, **46**: 211-217.

Frison, E.A. (1994). Sanitation techniques for cassava. *Tropical Science,* **34**: 146-153.

Gibbs, A. and Mackenzie, A. (1997). A primer pair for amplifying part of the genom of all potyvirids by RT-PCR. *Journal of Virological Methods,* **63**: 9-16.

Gibson, R.W. (1996). The report of a survey monitoring the spread of the epidemic of African cassava mosaic virus from Uganda into western Kenya. Internal report. Natural Resources Institute, Chatham, UK, 18p.

Gibson, R.W., Legg, J.P. and Otim-Nape, G.W. (1996). Unusual severe symptoms are characteristics of the current epidemic of mosaic virus disease in Uganda. *Annals of Applied Biology,* **128**: 479-490.

Gibson, R.W. and Otim-Nape, G.W. (1997). Factors determining recovery and reversion in mosaic-affected African cassava mosaic virus resistant cassava. *Annals of Applied Biology,* **131**: 259-271.

Gondwe, F.M.T., Mahungu N.M., Hillocks, R.J., Raya, M.D., Moyo, C.C., Soko, M.M., Chipungu, F.P., and Benesi, I.R.M. (2003). Economic Losses Experienced by Small-scale Farmers in Malawi Due to Cassava Brown Streak Virus Disease. In *Cassava Brown Streak Virus Disease*: Past, Present, and Future, J.P. Legg and R.J. Hillocks (eds). Aylesford, U.K.: Natural Resources International Limited. Proceedings of an International Workshop, 27-30

October 2002, Mombasa, Kenya. forthcoming. Washington, DC: International Food Policy Research Institute.

GriffithS, H.M.; Slack, S.A.; Dodds, J.H. (1990) Effect of chemical and heat therapy on virus concentrations in *in vitro* potato plantlets. *Canadian Journal of Botanic*, **68**: 1515-1521.

Hahn, B. H., Ebling, F., Freeman, ***S.,*** Clevinger, B., and Davie, J. (1980). Cassava mosaic disease resistance, cassava bacterial blight resistance and decreased cyanide content. *Arthritis Rheumatology*, **23:** 942-945.

Hahn, S.K., John, C., Isoba, G. and Ikotun, T. (1989). Resistance breeding in root and tuber crops at the IITA, Ibadan Nigeria. *Crop Protection,* **8**: 147-168.

Hahn, S.K. (1984). Progress of root and tuber improvement at IITA. In: *Proceedings of the 6th Symposium of the International Society for Tropical Root Crops* Lima, Peru 20-25 February 1983. Shideler, F. S. and Rincon, H. (eds.). 96p.

Hamilton, A.J. and Baulcombe, D.C. (1999). A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science,* **286**: 950-952.

Harrison, B.D., Swanson, M.M. and Robinson, D.J. (1995). Cassava viruses in the old world. In: proceedings of the second international scientific meeting, Bogor, Indonesia, CIAT working document. *Cassava Biotechnology Network*, **150**: 298-301.

Harpaz I and Cohen S. (1965). Semi persistent relationship between cucumber vein yellowing virus (CVYV) and its vector, the tobacco whitefly (*Bemisia tabaci* Gannadius). *Phytopathology,* **54**: 240-248.

Henry, G. and Hershey, C. (2002). Cassava in South America and the Caribbean. In: *Cassava: Biology, Production and Utilization*. Cambridge

University Press. CAB International, Wallingford, UK Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). 17p.

Herzberg, F. Mignouna, J. Mahungu, N.M. and Kullaya, A. (2004). Assessment of Genetic Diversity of Local Verieties of Cassava in Tanzania Using Molecular Markers. *African Crop Science Journal,* **12**:171.

Hillocks, R.J., Raya, M. and Thresh, M.J. (1996). The association between root necrosis and above ground symptoms of brown streak virus infection of cassava in southern Tanzania. *International Journal of pest management*, **42**: 285-289.

Hillocks, R.J. (1997). Cassava virus disease and their control with special reference to southern Tanzania. *International Journal of Pest Management*, **2**: 125-138.

Hillocks, R.J. and Thresh, J.M. (1998). Cassava mosaic and cassava brown streak virus diseases in Africa: a comparative guide to symptoms and aetiologies. Natural Resources Institute, UK. 11p.

Hillocks, R.J., Raya, M.D. and Thresh, J.M. (1999). Distribution and symptom expression of cassava brown streak virus disease in southern Tanzania. *African Journal of Root and Tuber Crops,* **3**: 57-62.

Hillocks, R.J., Raya, M., Mtunda, K. and Kiozia, H. (2001). Effects of cassava brown streak virus disease on yield and quality of cassava in Tanzania. *Journal of Phytopathology,* **149**: 389-394.

Hillocks, R.J. (2002). Cassava brown streak virus disease: Summary of present knowledge on distribution, spread, effect on yield and methods of control. In: *Proceedings of international workshop, Mombasa, Kenya*, 27-30 October 2002. Natural Resources International Limited, Aylesford, UK. 23p.

Hillocks, R.J., Thresh, J.M., Tomas, J., Botao, M. Macia, R. and Zavier, R. (2002). Cassava brown streak disease in northern Mozambique. *International Journal of Pest Management.* **48**: 179-182.

Hillocks, R.J. (2003). Cassava Brown Streak Virus Disease: Summary of Present Knowledge on Distribution, Spread, Effect on Yield and Methods of Control. In *Cassava Brown Streak Virus Disease: Past, Present, and Future,* ed. J.P. Legg and R.J. Hillocks. Aylesford, U.K.: Natural Resources International Limited. Proceedings of an International Workshop, 27-30 October 2002, Mombasa, Kenya.

Hillocks, R.J. and Jennings, D.L. (2003). Cassava brown streak disease: a review of present knowledge and research needs. *International Journal of Pest Management,* **43**: 225-234.

Hillocks, R.J. and Thresh, J.M. (2003). Cassava mosaic and cassava brown streak diseases in Nampula and Zambézia provinces of Mozambique. *Roots,* **8**: 10 -5

Hillocks, R.J. (2005). Promotion of control measures for cassava brown streak disease. Final Technical Report 01 August 2002 – 31 March 2005. R8404 (ZA 0632). Pp 2-31.

Hillocks, R.J. (2006). Promotion of control measures for cassava brown streak disease. Final Technical Report R8404 (ZA 0632). Pp 2-31.

Holn, T. (2007). Plant virus transmission from the insect point of view. *PNAS,* **104**: 17905-17906.

Hong, Y.G., Robinson, D.J., Harrison, B.D. (1993). Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted geminiviruses in cassava. *Journal of General Virology*, **74**: 2437-2443.

Hormozi-Nejad, M.H., Mozafari, J. and Rakhshanderoo, F. (2010). Production and certification of virus-free nucleus seeds incommon bean. *Journal of Plant Diseases and Protection*, **117:** 201-205.

Hu, J.S. (1995). Comparison of dot blot, ELISA and RT-PCR assays for detection of two *Cucumber mosaic virus* isolates infecting banana in Hawaï. *Plant Disease,* **79**: 902-906.

ICTV (2010). http://www.ictvonline.org/index.asp?bhcp=1 accessed on 14 02/2012.

Idris, I., Gray, S. and Donnelly, R. (2001). Protein kinase C activation: isozymespecific effects on metabolism and cardiovascular complications in diabetes. *Diabetologia* **44**: 659–673.

IITA (2005). Agronomy of cassava. IITA *Research Guide*s. Training Program, IITA, Ibadan, Nigeria. 39p.

James, D., Trytten, A., Mackenzie, D.J., Towers, G.H.N. and French, C.J. (1997). Elimination of apple stem grooving virus by chemotherapy and development of an immunocapture RT-PCR for rapid sensitive screening. *Annal of Applied Biology*, **131**: 459-470.

James, D., Varga, A., Pallas, V. and Candresse, T. (2006). Strategies for simultaneous detection of multiple plant viruses. *Journal of Plant Pathology,* **28**: 16-29.

Jameson, J.D. (1964). Cassava mosaic disease in Uganda. *East African Agricultural Journal,* **2**: 208-213.

Janssen, D., Martin, G., Velasco L., Gomez P., Segundo E., Ruiz L. and Cuadrado I.M. (2005). Absence of a coding region for the helper component-proteinase in the genome of the cucumber vein yellowing virus, a whitefly-transmitted member of the potyviridae. *Archives of Virology*, **150**: 1439-1447.

Jaramillo, G., Morante N., Perez, J.C., Calle, F., Ceballos, H., Arias, B., Bellotti, A.C. (2005). Diallel analysis in cassava adapted to the mid-altitude valleys environment. *Crop Science*, **45**: 1058–1063.

Jennings, D.L. (1957). Further studies in breeding cassava for virus resistance. *East African Agricultural Journal,* **22**: 213-219.

Jennings, D.L. (1960a). Observation on virus diseases of cassava in resistant and susceptible varieties. 1. Mosaic disease. *Empire Journal of Experimental Agriculture,* **28**: 23-34.

Jennings, D. L. (1960b). Observations on virus diseases of cassava in resistant and susceptible varieties. II. Brown streak disease. *Empire Journal of Experimental Agriculture,* **28**: 261-269.

Jennings, D.L. (1976). Breeding for resistance to African cassava mosaic disease. In: Report of Inter-disciplinary Workshop held at Mugugga, Kenya, 19-22 February 1976. IDRC-071e, Ottawa, Canada. Nestel, B.L. (ed.). Pp 39-44.

Jennings, D.L. and Iglesias, C. (2002). *Cassava: Biology, Production and Utilization*. CAB International, Wallingford, UK. Hillocks, R.J., Bellotti, A. and Thresh, J.M. (eds.). Pp 149-162.

Jennings D.L (2003). Historical perspective on breeding for resistance to cassava brown streak virus disease. Pp 55-57. In: Legg JP and Hillocks RJ (Eds), 2003. Cassava brown streak virus disease: Past, Present and Future. Proceedings of an international workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, UK. 100p.

Jeremiah, S.C. and Legg, J.P. (2008). Cassava brown streak virus disease: Farmers' perspectives on a new outbreak of this disease from the Lake zone of Tanzania in *www.youtube.com/watch?v=CJdws9CnUw:* accessed on 05/09/2011.

Jones, W.O. (1959). *Manioc in Africa.* Stanford University Press, Stanford, CA USA 314p.

Jones, A.L., Thomas, C.L. and Maule, A.J. (1998). *De novo* methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. *EMBO Journal* **17**: 6385-6393.

Jones, D.R. (2004). Plant viruses transmitted by whiteflies. *European Journal of plant Pathology*, **109**: 195-219.

Jose, G. and Dorea, F. (2004). Maternal Thiocyanate and Thyroid Status during Breast-Feeding. *Journal of the American College of Nutrition*, **23**: 97-101.

Kaiser, W.J and Teemba, L.R. (1989). Use of tissue culture and thermotheraphy to free East African cassava cultivars of African cassava mosaic virus and cassava brown streak diseases. *Plant Dis*ease *Reporter,* **63**: 780–784.

Kambewa, E. (2007). Assessing the feasibility and competitiveness of cassava-based ethanol production in the SADC countries: Preliminary results. In: *10th Triennial Symposium* jointly organized by the ISTRC-AB and Mozambique Agrarian Research Institute (IIAM). Joaquim Chissano International Conference Centre, Maputo, Mozambique 8-12 October 2007. Mahungu, M.N. and Mkumbira, J (eds.) 72p.

Kanju, E. (1989). Safari report to Masasi, 20-24 August 1989. Naliendale Agricultural Research Institute, Mtwara Tanzania. 3p.

Kanju, E., Mahungu, N., Dixon, A. and Whyte, J. (2003a). Is resistance/tolerance of cassava to brown streak disease associated with the zigzag stem trait? *Roots,* **8**:15-18.

Kanju, E.E, Mtunda, K.J, Muhana, M., Raya, M.D, and Mahungu, N.M. (2003b). Management of cassava brown streak virus disease in Tanzania Pp 66-69. In: Legg JP and Hillocks RJ (Eds.), 2003. Cassava Brown Streak Virus Disease: Past, Present and Future. Proceedings of an International Workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, UK. 100p.

Karamagioli, M. (1994). Molecular detection and characterization of African cassava mosaic virus and Cassava brown streak virus. Msc Dessertation, Department of Agriculture University Of Reading, U.K.

KARI, (1983). Scientist in East Africa looks for ways to combat virus attacking cassava crops. Kenyan Agricultural Research Institute Newsletter. 23p.

Kartha, K.K. and Gamborg, O.L. (1975). Elimination of cassava mosaic disease by meristem culture. *Phytopathology*, **65**: 826-828.

Kartha, K.K. (1981). *Meristem culture and cryopreservation methods and applications in Plant Tissue Culture*: Methods and Applications in Agriculture. Thorpe, T.A. (ed.), Academic Press, New York. Pp181-211.

Kassanis, B. (1957). The Use of Tissue Culture to Produce Virus-free Potato Varieties. *Annal of applied Biology*, **45**: 422-427.

Kasschau, K.D. and Carrington, J.C. (1998). A counter defensive strategy of plant viruses: suppression of post-transcriptional gene silencing. *Cell*, **95**: 461-470.

Katinila, N.A., Hamza, H. and Raya, M.D. (2003). Social Impact of Cassava Brown Streak Virus Disease on Rural Livelihoods in Southern Tanzania. Pp 46-52. In: Legg JP and Hillocks 192 RJ (Eds), 2003. Cassava brown streak virus disease: Past, Present and Future. Proceedings of an international

workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, Uk. 100pp.

Kawano, K. (2003). Thirty years of cassava breeding for productivity: biological and social factors for success. *Crop Science*, **43**: 1325-1335.

Kheyr-Pour, A., Gronenborn, B. and Czosnek, H. (1994). Agroinoculation of tomato yellow leaf curl virus (TYLCV) overcomes the virus resistance of wild Lycopersicon species. *Plant Breeding*, **112**: 228–233.

Kitajima, E.W. and Costa, A.S. (1964). Elongated particles found associated with *Cassava brown streak virus*. *East African Agriculture and Forestry Journal,* **29**: 28-30.

Klein, R.E. and Livingston C.H. (1982). Eradication of potato virus X from potato by ribavirin treatment of culture potato shoot tips. *American Potato Journal*, **59**: 359-365.

Ko, C.C. (2001). Systemic of Aleyrodidae- review and prospection. In: Proceedings of the Symposium on the progress of Taiwan Entomological Research at the Threshhold of 21st Century in Taichung. Pp 192 -199.

Kokkinos, C.D. and. Clark, C.A (2006). Real-Time PCR Assays for Detection and Quantification of Sweetpotato Viruses. *Plant Disease*, **90**: 783-788.

Korean, J. (2003). Micropropagation of cassava suspension culture derived from node explants. *Plant Tissue Culture,* **27**: 185-189.

Kreuze, J.F., Savenkov, E.I. and Valkonen, J.P.T. (2002). Complete genome sequence and analysis of the subgenomic RNAs of *Sweet potato chlorotic stunt virus* reveal several new features for the genus *Crinivirus*. *Journal of Virology,* **76**: 9260-9270.

Llave, C., Xie, Z., Kasschau, K.D. and Carrington, J.C. (2002). Cleavage of *Scarecrow*-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science,* **297**: 2053-2060.

Lapidot, M. & Friedmann, M. (2002). Breeding for resistance to whitefly-transmitted geminiviruses, *Annal of Applied Bioliology*, **140**: 109-127.

Lava Kumar, P., Akinbade, S.A., Dixon, A.G.O., Mahungu, N.M., Mutunda, M.P., Kiala, D., Londa, L., Legg, J.P. (2009). First report of the occurrence of East African cassava mosaic virus-Uganda (EACMV-UG) in Angola. *Plant Pathology*, **58**: 402.

Lecoq, H., Desbiez, C., Delécolle, B., Cohn, S. and Mansour, A. (2000). Cytological and molecular evidence that the whitefly-transmitted *Cucumber vein yellowing virus* is a tentative member of the family *Potyviridae. Journal of General Virology,* **81**: 2289-2293.

Legg, J.P. (1994). *Bemisia tabaci*: the whitefly vector of cassava mosaic Geminiviruses in Africa: An ecological perspective. *African Crop Science Journal*, **4**: 437-448.

Legg, J.P., Gibson, R.W. and Otim-nape, G.W. (1994). Genetic polymorphism amongst Ugandan populations of *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae), vector of African cassava mosaic geminivirus. *Tropical Science,* **34**: 73-81.

Legg, J.P. and Raya, M. (1998). A survey of cassava virus disease in Tanzania. *International Journal of Pest Management,* **44**:17-23.

Legg, J.P. and Hillocks, R.J. (Eds), (2003). Cassava Brown Streak Virus Disease: Past, Present and Future. Proceedings of an International Workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, UK. 100p.

Legg, J.P. and Thresh, M.J. (2003). Cassava virus disease in Africa. In: *Proceedings of the first International Workshop on Plant Virology* in Sub-Saharan Africa, 4-8 June 2003 Ibadan, Nigeria. IITA Publications Pp 517-552.

Legg, J.P., Jeremiah, S.C., Obiero, H.M., Maruthi, M.N., Ndyetabula, I., Okao-Okuja, G., Bouwmeester H., Bigirimana, S., Tata-Hangy, W., Gashaka, G., Mkamilo, G., Alicai, T. and Kumar, L.P. (2011). Comparing the regional epidemiology of the cassava mosaic and cassava brown streak virus pandemics in Africa. *Virus Research*, **159**: 161-170.

Leihner, D. (2002). Agronomy and cropping systems. In: *Biology, Production and Utilization*. Cambridge University Press. CAB International, Wallingford, UK Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). Pp 23-27.

Lennon, A.M., Aiton, M.M. and Harrison, B.D.H. (1986). *Cassava viruses from Africa.* Report for 1985, Scottish Crop Research Institute. 168. Dundee, Scotland: SCRI Wallingford, UK. 12p.

Leonhard W., Wawrosch, C.H., Aue, R.A. and Kopp, B. (1998). Monitoring of virus diseases in Austrian grapevine varieties and virus elimination using *in vitro* thermotherapy. *Plant Cell Tissue and Organ Culture*, **52**: 71-74.

Leone, J. (1977). Origin, evolution and early dispersal of root and tuber crops. In: *Proceedings of the 4th Symposium of the International Society of Tropical Root Crops* held at CIAT, Cali, Colombia 1-7 August, 1976. IDRC-080e Ottawa, Canada. Cock, J.H., McIntyre, R. and Graham, M. (eds.). Pp 20-30.

Li, H.W., Lucy, A.P., Guo, H.S., Li, W.X., Ji, L.H., Wong, S.M. and Ding, S.W. (1999). Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. *The EMBO Journal*, **18**: 2683-2691.

Li, W., Hilf, M.E., Webb, S.E., Baker, C.A. and Adkins, S. (2008). Presence of P1b and absence of HC-Pro in *Squash vein yellowing virus* suggests a

general feature of the genus *Ipomovirus* in the family *Potyviridae. Virus Research*, **135**: 213-219.

Lister, R.M. (1959). Mechanical transmission of *Cassava brown streak virus*. *Nature,* **183**: 1588-1589.

Livak, K. and Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. *Methods* **25**: 402-408.

Lizarraga, R.E., Salazar, L.F., Roca, W.M. and Schilde-Rentschler, L. (1980). Elimination of *Potato spindle viroid* by low temperature and meristem culture. *Pathology* **70**: 754-755.

Lodhi, M. A., Ye, G. N., Weeden, N. F. and Reisch, B. I. (1994). A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Plant Molecular Biology Reporter*, **12**: 6–13.

MacRae, J. and Mukesh, S. (1998). Detection of viruses by electron microscopy: An efficient approach. *Journal of Virological Methods,* **72**:105-108.

Mahfouze; S.A., El-Dougdoug, and K A. and. Allam, E.K. (2010). Production of *Potato Spindle Tuber Viroid*-Free Potato Plant Materials *in Vitro. Journal of American Science,* **6**: 1570-1577.

Mahungu, N.M., Bidiaka, M., Tata., H., Lu-Kombo, S. and N'luta, S. (2003). Cassava brown streak-like symptoms in Democratic Republic of Congo. *Roots,* **8**: 8-9.

Malauri, B.; Trouslot, M.; Bertraud, J.; Bousalem, M.; Pinel, A. and Dubern, J. (1998). Medium-term and long-term *in vitro* conservation and safe international exchange of yam (*Dioscoria* spp.) germplasm. *Electronic Journal of Biotechnology*, **1**:1-15.

Mantell, S.H., Haque, S.Q. and Whitehall, A.P. (1980). Apical meristem tip culture for eradication of flexous rod viruses in yams (Dioscorea alata). *Tropical Pest Management*, **26**: 170-179.

Mansour, A. and Al-Musa, A. (1993). Cucumber vein yellowing virus; host range and virus vector relationships. *Journal of Phytopathology*, **137**: 73-78.

Manzer, F.E and Merriam, D. (1961). Field transmission of the potato spindle tuber virus and virus X by cultivating and hilling equipment. *American Potato Journal,* **38**: 346-352.

Martin, J. H., Mifsud, D. and Rapisarda, C. (2000). The whiteflies (Hemiptera; Aleyrodidae) of Europe and the Mediterranean basin. *Bulletin of Entomological Research,* **90**: 407-448.

Maruthi, M.N., Colvin, J. and Seal, S. (2001). Mating compatibility, life-history traits and RAPD-PCR variation in *Bemisia tabaci* associated with the cassava mosaic disease pandemic in East Africa. *Entomologia Experimentalis et Applicata*, **99**: 13-23.

Maruthi, M.N., Colvin, J., Seal, S., Gibson, G. and Cooper, J. (2002). Co-adoptation between cassava mosaic geminiviruses and their local vector populations. *Virus Research,* **86**: 71-85.

Maruthi, M.N., Hillocks, R.J., Mtunda, K., Raya, M.D., Muhanna, M., Kiozia, H., Rekha, A.R., Colvin, J. and Thresh, J.M. (2005). Transmission of *Cassava brown streak virus* by *Bemisia tabaci*. *Journal of Phytopathology,* **153**: 307-312.

Mathews, R.E.F. (1991). *Plant virology*, second edition, Academic Press New York. 23p.

Matthews, R.E.F. (2002). *Plant Virology,* Fourth Edition, Academic Press Inc.,UK. 835p.

Mbanzibwa, D.R., Tian, Y.P., Tugume, A.K., Mukas, S.B., Tairo, F., Kyamanywa, S., Kullaya, A., Jari, P. and Volkonen, T. (2009). Genetically distinct strains of Cassava brown streak virus in the Lake Victoria basin and the Indian Ocean coastal area of East Africa. *Archives of Virology*, **154**: 353-359.

Mbanzibwa, D.R., Tian, Y.P., Tugume, A.K., Patil, B.L., Yadav, J.S., Bagewadi, B., Abarshi, M.M., Alicai, T., Changadeya, W., Mkumbira, J., Muli, M.B., Mukasa, S.B., Tairo, F., Baguma, Y., Kyamanywa, S., Kullaya, A., Maruthi, M.N., Fauquet, C.M., Valkonen, J.P.T. (2011). Evolution of cassava brown streak disease-associated viruses. *Journal of General Virology,* **92**: 974-987.

McSween, S., Walker, T., Salegua, V. and Pitoro, R. and (2006). Economic Impact on Food Security of Virietal Tolerance to Cassava Brown Streak Disease in Costal Mozambique. Research Report No. 1E August 2006. Pp 2-30.

Meybodi, D.E. Mozafari, J. Babaeiyan, N. and Rahimian, H. (2011). Application of Electrotherapy for the Elimination of Potato Potyviruses. *Journal of Agricultural Science Technology*, **13**: 921-927.

Meyer, H. (1984). Les Barundi: Une étude ethnologique en Afrique orientale. Société Française d'Histoire d'Outre-Mer, Paris.

Mhone, A., Sandifolo, V., Jumbo, S. and Mahungu, N. (2007). Cassava based ethanol: could it compromise food security? In: *10th Triennial Symposium* jointly organized by the ISTRC-AB and Mozambique Agrarian Research Institute (IIAM). Joaquim Chissano International Conference Centre, Maputo, Mozambique 8-12 October 2007. Mahungu, M.N. and Mkumbira, J. (eds.) 21p.

Mkamilo, G.S. and Jeremiah, S.C. (2005). Current status of cassava improvement programme in Tanzania. *African Crop Science*, **7**: 1311-1314.

Mlingi, N.L.V., Nkya, S., Tatala, S.R., Rashid, S. and Bradbury, J.H. (2011). Recurrence of konzo in southern Tanzania: Rehabilitation and prevention using the wetting method. *Food and Chemical Toxicology,* **49**: 673-677.

Moissiard, G. and Voinnet, O. (2004). Viral suppression of RNA silencing in plants. *Molecular Plant Pathology,* **5**: 71-82.

Monger, W.A., Seal, S., Cotton, S. and Foster, G.D. (2001a). Identification of different isolates of *Cassava brown streak virus* and development of a diagnostic test. *Plant Pathology,* **8**: 768-775.

Monger, W.A., Seal, S., Isaac, A. M. and Foster, G.D. (2001b). Molecular characterisation of cassava brown streak virus coat protein. *Plant Pathology,* **50**: 527-534.

Monger, W.A., Alicai, T., Ndunguru, J., Kinyua, Z.M., Potts, M., Reeder, R.H., Miano, D.W., Adams, I.P., Boonham, N., Glover, R.H. and Smith, J. (2010). The complete genome sequence of the Tanzanian strain of Cassava brown streak virus and comparison with the Ugandan strain sequence. *Archives of Virology*, **155**: 429-433.

Morales, F.J. and Anderson, P.K. (2001). The emergence and dissemination of whitefly transmitted viruses in Latin America. *Archives of Virology,* **146**: 415-441.

Morel, G. and Martin, C. (1952). Guerison de dahlias atteints d'ume Maladie a Virus. *Comptes Rendus de l Academie des Sciences*, **235**: 1324-1325.

Moreno, I., Gruissem, W. and Vanderschuren, H. (2011). Reference genes for reliable potyvirus quantification in cassava and analysis of cassava brown streak virus load in host varieties. *Journal of Virological Methods*, **177**: 49-54.

Mound, L.A. (1983). Biology and identity of whitefly vectors of plant pathogens. In: Plumb, R.T. and Thresh, J.M., Editors, 1983. *Plant Virus Epidemiology*, Blackwell, Oxford, UK, Pp 305–313.

Mtunda, K. Mahungu, N.M., Thresh, J.M., Kilima, M.S. and Kioza, H.C. (1998). Phytosanitation involving the use of 'clean' planting material for the control of cassava brown streak virus disease. S.R.I, SARRNET and NRI Greenwich University Chatham United Kingdom. Pp 1 -5.

Mtunda, K.J., Muhanna, M., Raya, M.D., and Kanju, E.E. (2003). Current Status of Cassava Brown Streak Virus Disease in Tanzania. In *Cassava Brown Streak Virus Disease: Past, Present, and Future,* J.P. Legg and R.J. Hillocks (eds). Aylesford, U.K.: Natural Resources International Limited. Proceedings of an International Workshop, 27-30 October 2002, Mombasa, Kenya.

Muhanna, M. and Mtunda, K. (2003). Report on the study of cassava root rot problem in Muheza District, Tanga Region, Tanzania. Submitted to the Tanga District Director May 2002. 23p.

Mukasa, S.B., Rubaihayo, P.R. and Valkonen, J.T.P. (2003). Incidence of viruses and viruslike disease of sweetpotato in Uganda. *Plant Disease*, **87**: 329-335.

Mumford, R.A., Boonham, N., Tomlinson, J. and Barker, I. (2006). Advances in molecular phytodiagnostics: New solutions for old problems. *Journal of Plant Pathology,* **116**: 1-19.

Munga, T. and Thresh, M.J. (2002). The incidence of cassava mosaic and cassava brown streak diseases in coastal Kenya. Project Report, Natural Resources Institute, Chatham, UK. 21p.

Muniyappa, V. (1980). Whiteflies. In: *Vectors of Plant Pathogens*. 1980. Academic Press, New York. Harris, K.F. and Maramorosch, K. (eds.). Pp. 39-85.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiologia Plantarum* **15**: 473-497.

Mware, B., Narla, R., Amata, R., Olubayo, F., Songa, J. Kayamanua, S. and Ateka, E.M. (2009). Efficiency of *cassava brown streak virus* transmission by two whitefly species in coastal Kenya. *Journal of General and Molecular Virology,* **1**: 40-45.

Nascimento, L.C., Pio-Ribeiro, G., Willadino, L. and Andrade, G.P. (2003). Stock indexing and *potato virus y* elimination from potato plants cultivated *in vitro. Scientia Agricola*, **60**: 525-530.

Nassar, N.M.A. (1995). Development of cassava interspecific hybrids for savanna (cerredo) conditions. *Journal of Root Crops,* **22**: 9-17.

Nassar N.M.A., (2002). Cassava, *Manihot esculenta* Crantz, genetic resources: Origin of the Crop, its evolution and relationship with wild relatives. *Genetic and Molecular Research,* **1**: 298-305.

Nassar, N.M.A. and Ortiz, R. (2010). Cassava improvement: challenges and impacts, *Journal of Agricultural Science,* **145**: 163-171.

Nassuth, A., Pollari, E., Helmeczy, K., Stewart, S. and Kofalv, S.A. (2000). Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. *Journal of Virology Methods*, **90**: 37–49.

Nault, L.R. (1997). Arthropod transmission of plant viruses: a new synthesis. *Annals of Entomological Society of America,* **90**: 522–41.

Ng, K.C., Handley, C.J., Preston, B.N. and Robinson, H.C. (1992). The extracellular processing and catabolism of hyaluronan in cultured adult articular cartilage explants. *Archieve Biochemical Biophysiology,* **298**: 70-79.

Ng, J.C.K. and Falk, B.W. (2006). Virus-vector interactions mediating non-persistent and semi persistent transmission of plant viruses. *Annual Review of Phytopathology*, **44**:183-212.

Nichols, R.F. (1947). Breeding cassava for virus resistance. *East African Agricultural Journal,* **12**: 184-194.

Nichols, R.F. (1950). The brown streak disease of cassava; distribution climatic effects and diagnostic symptoms. *East African Agricultural Journal,* **15**: 154-160.

Nicot, N., Hausman, J., Hoffmann, L.and. Evers, D. (2005). Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *Journal of Experimental Botany*, **56**: 2907-2914.

Njock, T.E., Atiri, G.I., Thottappilly, G. and Thresh, J.M. (1996). Disease development and recovery in resistant and susceptible cassava genotypes infected by African cassava mosaic geminivirus. *African Plant Protection*, **2**: 97-102.

Ntawuruhunga, P., Ssemakula G., Ojulong H., Bua A., Ragama P., Kanobe C.and Whyte J. (2006). Evaluation of advanced cassava genotypes in Uganda. *African Crop Science Journal,* **14**: 15-27.

Ntawuruhunga, P. and Legg, J. (2007). Spread of cassava brown streak virus disease and its implications for the movement of cassava Germplasm in the east and central Africa region. Joint collaboration paper USAID and IITA under Crop Crisis Control Project. 43p.

Nweke, F.I., Dixon, A.G.O., Asiedu, R. and Folayan, S.A. (1994). Cassava varietal needs of farmers and the potential for production growth in Africa. Working paper No. 10. Collaborative Study of Cassava in Africa. IITA, Ibadan. Pp220-238.

Nweke, F.I., Spencer, D.S.C. and Lynam, J.K. (Eds.) (2002). *The Cassava Transformation.* East Lansing: Michigan State University Press. 61p.

Nweke, F.I. (2003). New challenge in the cassava transformation in Nigeria and Ghana. Paper presented at the InWEnt, IFPRI,NEPAD,CTA. 'Successes in African Agriculture'. 12p.

Nyland, G. and Goheen, A.C. (1969). Heat therapy of virus diseases of perennial plants. *Annual Review of Phytopathology*, **7**: 331-354.

Nzwalo, H. and Cliff J. (2011). Konzo: From Poverty, Cassava, and Cyanogen Intake to Toxico-Nutritional Neurological Disease. *Review*, **5**: e1051.

Ogbe, F.O., Dixon, A.G.O., Atiri, G.L. and Thottappilly, G. (2002). Restriction of virus movement into axillary bud is an important aspect of resistance in cassava to *African cassava mosaic virus. Journal of Phytopathology,* **150**:546-552.

Ogwok, E., Patil, B. L., Alicai, T. and. Fauquet, C.M. (2010). Transmission studies with Ugandan Cassava brown streakvirus (Potyviridae: Ipomovirus) and its interaction with abiotic and biotic factors in *Nicotiana benthamiana*. *Journal of Virological Methods*, **169**: 296-304.

Okogbenin, E., Porto, M.C.M., Egesi, C., Mba, C., Espinosa, E., Santos, L.G., Ospina, C., Marin, J., Barrera, E. and Gutierrez, J. (2007). Marker-Assisted Introgression of Resistance to Cassava Mosaic Disease into Latin American Germplasm for the Genetic Improvement of Cassava in Africa. *Crop Science Journal,* **47**: 1895-1904.

Otim-Nape, G.W. (1990). Root Crops in Uganda. In: Hartmans (ed). Agricultural research in Uganda. Five year research plan, USAID, Kampala. 12p.

Otim-Nape, G.W., Bua, A., Baguma, Y. (1994). Accelerating the transfer of improved crop production technologies: controlling African cassava mosaic virus disease in Uganda. *African Crop Science Journal*, **2**: 479-495.

Otim-Nape, G.W., Bua, A., Thresh, J.M., Baguma, Y., Ogwal, S., Semakula, G.N., Acola, G., Byabakama, B., Martin, A. (1997). Cassava Mosaic Virus Disease in Uganda: The Current Pandemic and Approaches to Control. Natural Resources Institute, Chatham, UK. 3p.

Otim-Nape, G.W., Alicai, T. and Thresh, J.M. (2001). Changes in the incidence and severity of cassava mosaic virus disease, varietal diversity and cassava production in Uganda. *Annals of Applied Biology,* **138**: 313-327.

Owor, B., Legg, J.P., Okao-Okuja, G., Obonyo, R. and Ogenga-Latigo, M.W. (2005). The effect of cassava mosaic geminiviruses on symptom severity, growth and root yield of a cassava mosaic virus disease-susceptible cultivar in Uganda. *Annals of Applied Biology,* **145**: 331-337.

Pacumbaba, R.P. (1985). Virus-free shoots from cassava stem cuttings infected with cassava latent virus. *Plant Disease,* **69**: 231-232.

Pasch, H. (1980). Linguistische Aspekte der Verbreitung Lateina merika nischer Nutzpflanzen in Afrika. *Unpublished PhD thesis, University of Cologne, Cologne, Germany.* Pp 23-45.

Patil, B.L, Ogwok, E., Wagaba, H., Mohammed, I.U., Yadav, J.S., Bagewadi, B., Taylor N. J., Kreuze, J., Maruthi, M. N., Alicai, T. and Fauquet, C. (2010). RNAi-mediated resistance to diverse isolates belonging to twovirus species involved in Cassava brown streak disease. *Molecular Plant Pathology,* **12**: 31-41.

Patino, M.T. (2007). Economic feasibility for producing ethanol from cassava in selected African countries. In: *10th Triennial Symposium* jointly organized by the ISTRC-AB and Mozambique Agrarian Research Institute (IIAM).

Joaquim Chissano International Conference Centre, Maputo, Mozambique 8-12 October 2007. Mahungu, M.N. and Mkumbira, J (eds.). 72p.

Paximadis, M., Idris, A.M., Torres-Jerez, I., Villarreal, I., Rey, M.E.C. and Brown, J.K. (1999). Characterization of geminiviruses of tobacco in the Old and New World. *Archives of Virology,* **144**: 703-717.

Pearce, F. (2007). Africa's cassava comeback. New Scientist, Environment. Magazine issue 2600.**http://www.newscientist. com/ article/ mg19426002 .000-africas -cassava-comeback.html**. Accesssed 24/09/2010.

Perring, T.M. (2001). The *Bemisia tabaci* species complex. *Crop Protection,* **20**: 725-737.

Pirone, T.P. (1981). Efficiency and selectivity of the helper component mediated aphid transmission of purified potyviruses. *Phytopathology*, **71**: 922–24.

Pirone, T. P. and Thornbury, D. W. (1988). Quantity of virus required for aphid transmission of a potyvirus. *Phytopathology,* **78**: 104-107.

Pita, J.S., Fondong, V.N., Sangare, A., Otim-Nape, G.W., Ogwal, S. and Fauquet, C.M. (2001). Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. *Journal of General Virology,* **82**: 655- 665.

Plucknett, L.D. (1984). Tropical Root Crops in the Eighties In: *Symposium of International Society for Tropical root crops*, 6th Lima, 20-25 February 1983. Shideler, F. S. and Rincon, H. (eds.). 3p.

Polston, J.E. and Anderson, P.K. (1997). The emergence of whitefly-transmitted geminiviruses in tomato in the Western hemisphere. *Plant Disease,* **81**: 1358–1369.

Purseglove, J.W. (1968). *Tropical Crops*: Dicotyledons, Longman, London, UK. 56p.

Quak, F. (1961). Heat treatment and substances inhibiting virus multiplication in meristem culture to obtain virus free plants. In: Proceedings 25 International Horticultural Congress, **1**: 144-155.

R Development Core Team (2010). R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org. Accessed on 23/05/2010.

Raspisarda, C. and TropeaGarzia, G. (2002). Tomato yellow leaf curl Sardinia virus and its vector *B. tabaci* in Sicilia, Italy: present status and control possibilities. *EPPO Bulletin,* **32**: 25-29.

Ratcliff, F., Harrison, B.D and Baulcombe, D.C., (1997). A similarity between viral defense and gene silencing in plants. *Science,* **276**: 1558-1560.

Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D.C. (1999). Gene silencing without DNA: RNA-mediated cross-protection between viruses. *Plant Cell,* **11**: 1207-1216.

Rhoades, R.E. (1996). The art of the informal agricultural survey. IITA *Research Guide 36*. Training Program, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. 2nd edition. 57p.

Robertson, I.A.D. (1987). The whitefly, *Bemisia tabaci* (Gennadius) as a vector of African cassava mosaic virus at the Kenya coast and ways in which the yield losses in cassava *Manihot esculenta* Crantz caused by the virus can be reduced. *Insect Science Application,* **8**: 797-801.

Roca, W.M.; Rodrígues, J.A.; Mafla G. and Roa, J. (1984). Procedures for recovering cassava clones distributed *in vitro*. CIAT, Colombia. 21p.

Rogers, D.J. and Appan, S.G. (1973). Manihot, Manihotoides (Euphorbiaceae). Flora Neotropica, Mongraph 13, Hafner Press, New York. 12p.

Rogers, D.J. (1963). Studies of M. esculenta Crantz and related species. *Bulletin of the Torry Botanical Club*, **90**: 43-54.

Ross, H.B. (1975). The diffusion of the manioc plant from South America to Africa: An essay in ethnobotanical culture history. *PhD dissertation. Faculty of Political Science, Columbia University, New York, USA.* 135p.

Rossel, H.W., Asiedu, R. and Dixon, A.G.O. (1992). Resistance of cassava to African cassava mosaic virus: what really happens. *Tropical Root and Tuber Crops Bulletin*, **6**: 2.

Rossel, H.W., Changa, C.M. and Atiri, G.I. (1994). Quantification of resistance to African cassava mosaic virus in IITA- improved mosaic-resistant breeding materials. In: *Proceedings of the 5th Symposium of the International Society for Tropical Root Crops. Root crops for food security in Africa.* Africa branch. IITA, Ibadan, Nigeria.Akoroda, M.O. (ed.). Pp 280-287.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den Hoff, M.J.B. and Moorman, A.F.M. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research,* **37**: 41-45.

Ruiz, M.T., Voinnet, O. and Baulcombe, D.C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell,* **10**: 937-946.

Salcedo1, A., Valle1, A.D., Sanchez1, B., Ocasio1, V., Ortiz1, A., Marquez, P., and Siritunga, D. (2010). Comparative evaluation of physiological post-harvest root deterioration of 25 cassava (*Manihot esculenta*) accessions: visual vs hydroxycoumarins fluorescent accumulation analysis. *African Journal of Agricultural Research,* **5**: 3138-3144.

Samuel, G. (1934). The movement of Tobacco mosaic virus within the plant. *Annals of Applied Biology*, **21**: 90-111.

Sarver, N. and Stollar, V. (1978). Virazole prevents production of Sindbis virus and virus-induced cytopathic effect in Aedes albopictus cells. *Virology,* **91**:267-282.

Sauti, R.F.N. and Chipungu, F.P. (1993). *Root and Tuber Crops Annual Report,* Bvumbwe Research Station, Limbe Malawi. 13p.

Scott, G.J., Rosegrant, M. and Ringler, C. (2000). Roots and tubers for the 21st century: Trends, projections and policy options. Food, Agriculture and the Enviornment Discussion Paper 31. Washington, D.C.: International food policy research institute (IFPRI) and International potato centre (CIP)

Shaba, E.R., Chipungu, F. and Mazuma, E.D.L. (2003). Cassava brown streak virus disease in Malawi. Pp 18-19. In: Legg JP and Hillocks RJ (Eds), 2003. Cassava Brown Streak Virus Disease:Past, Present and Future. Proceedings of an International Workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, UK. 100p.

Slack, J.M.W., Darlington, B.G., Heath, J.K. and Godsave, S.F. (1987). Mesoderm induction in early Xenopus embryos by heparin-binding growth factors *Nature*, **326**: 197-200.

Solomon-Blackburn, R.M. and Baker. H. (2001). Breeding virus resistant potato (*Solanum tuberosum*): A review of traditional and molecular approaches. *Heredity,* **86**:17 -35.

Stace-Smith, R. and Mellor, F.C. (1968). Eradication of potato viruses X and S by thermotherapy and axillary bud culture. *Phytophatology*, **58**: 199-203.

Steel, J.A.C. (2003). Cassava brown streak virus disease management in Mozambique: the NGOs' Perspective. Pp 70-72. In: Legg JP and Hillocks RJ (Eds), 2003. Cassava Brown Streak Virus Disease: Past, Present and Future.

Proceedings of an International Workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, Uk. 100p.

Storey, H. H. (1936). Virus diseases on East African plants - VII. A progress reports on studies of diseases of cassava. *East African Journal,* **2**: 34-39.

Storey, H.H. (1938). Cassava virus diseases. East African Agricultural Research Station, Amani, Tanzania. Tenth Annual Report. Majesty's stationary office, London, UK. Pp 10- 13.

Storey, H.H. and Nichols, R.F. (1938). Studies of the mosaic disease of cassava. *Annals of Applied Biology,* **25**: 790-860

Storey, H.H. (1939). Report of the Plant Pathologist of the East African Agricultural Research Station for 1939. Amani, Tanzania. 34p.

Storey, H.H. (1947). Virus diseases on East African plants-III- Plant Pathology Reports. East African Research Institute. Amani, Tanzania. Pp 8-9.

Swanson, M.M. and Harrison, B.D. (1994). Properties, relationships and distribution of cassava mosaic geminiviruses. *Tropical Science*, **34**: 15-25.

Takeshima, H. (2010). Prospect for development of genetically modified cassava in Sub-Saharan Africa. *AgbioForum*, **13**: 63-75.

Taylor, N., Chavarriaga, P., Raemakers, K., Siritunga, D. and Zhang, P. (2004). Development and application of transgenic technologies in cassava. *Plant Molecular Biology*, **56**: 671-688.

Thomas, P. (2006). Re-emergence of covert bacteria *Bacillus pumilus* and *Brevibacillus* sp. in microbe-freed grape and watermelon stocks attributable to occasional autoclaving-defying residual spores from previous cycles. *Plant Cell Tissue Organells Cul*ture, **87**: 155–165.

Thottappilly, G., Thresh, J.M., Calvert, L.A., and Winter S, (2003). Cassava. in: Virus and virus-like diseases of major crops in developing countries. Loebenstein G.and Thottappilly, G. (eds.) Kluwer Academic Publ., Dordrecht, the Netherlands. Pp107-165.

Thresh, J.M., Fargette, D. and Otim-Nape, G.W. (1994). The viruses and virus disease of cassava in Africa. *African Crop Science Journal,* **2**: 459-478.

Thresh, J.M., Otim-Nape, G.W., Thankapan, M. and Muniyappa, V. (1998). The mosaic diseases of cassava in Africa and India caused by whitefly-borne geminiviruses. *Review of Plant Pathology,* **77**: 935-945.

Thresh, J.M, (2003). Brief History of cassava brown streak virus disease. *In*: Legg JP and Hillocks RJ (Eds), 2003. Cassava Brown Streak Virus Disease: Past, Present and Future. Proceedings of an international workshop, Mombasa, Kenya, 27-30 October, 2002. Natural Resources International Limited, Aylesford, UK. 100pp.

Thresh, J.M. and Hillocks, R.J. (2003). Cassava mosaic and cassava brown streak diseases in CBSV-[MZ:Nam1-1:07] and Zambezia Provinces of Mozambique. *Roots*, **8**: 10-15.

Thresh, J.M. and Cooter, R.J. (2005). Strategies for controlling cassava mosaic virus disease in Africa. *Plant Pathology,* **54**: 587–614.

Valentine, T.A., Roberts, I.M. and Oparka, K.J. (2002). Inhibition of tobacco mosaic virus replication in lateral roots is dependent on an activated meristem-derived signal. *Protoplasma,* **219:** 184-196.

Van den Bosch, F., Jeger, M.J. and Gilligan, C.A. (2007). Disease control and its selection for damaging plant virus strains in vegetatively propagated staple food crops. *Proceedings of the Royal Society: Biological Journal*, **274**: 11–18.

Vaucheret, H. and Fagard, M. (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends in Genetics,* **17**: 29-35.

Verma, P.M. (1963). Transmission of plant viruses by whiteflies. *Bulletin of Natural Science,* **24**: 11-33.

Verma, N., Raja, R., Hallan, V., Kumar, K., Zaidi, A.A. (2004). Production of *Cucumber mosaic virus*-free chrysanthemum by meristems tip culture. *Crop Protection*, **23**: 469-473.

Vidavsky, F. and Czosnek, H. (1998). Tomato breeding lines resistant and tolerant to tomato yellow leaf curl virus issued from Lycopersicon hirsutum. *Phytopathology*, **88**: 910-914.

Voinnet, O., Lederer, C. and Baulcombe, D.C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana. Cell,* **103**: 157-167.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends in Genetics,* **17**: 449-459.

Walkey, D.G.A. (1976). High temperature inactivation of *Cucumber* and *Alfalfa mosaic viruses* in *Nicotiana rustica* cultures. *Annals of Applied Biology.* **84**: 183-192.

Walkey, D.G.A. and Freeman, G.H. (1977). Inactivation of *Cucumber mosaic virus* in cultured tissues of *Nicotiana rustica* by diurnal alternating periods of high and low temperature. *Annals of Applied Biology,* **87**: 375-382.

Walkey, D.G.A. (1985). *Applied Plant Virology*. New York: Wiley Interscience. Pp 237-241.

Wasswa, P., Alicai, T. and Mukasa, S.B. (2010). Optimisation of *in vitro* techniques for cassava brown streak virus elimination from infected cassava clones. *African Crop Science Journal*, **18**: 235-241.

Waterhouse, P.M, Wang, M.B. and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature,* **411**: 834-842.

Watson, H.A. and Roberts, F.H. (1939). A comparative study of the transmission of Hyoscyamus virus 3, potato virus Y, and cucumber virus 1 by the vectors *Myzus persicae* (Sulz.), *M. circumflexus* (Buckton) and *Macrosiphum gel* (Koch). In: *Proceedings of the Royal Society of London, Series B*, **127**: 543-576.

Weimin, L., Mark, E.H., Susan, E.W., Carlye, A.B. and Scott, A. (2008). Presence of P 1b and absence of HC-Pro in Squash vein yellowing virus suggests a general feature of the genus *Ipomovirus* in the family *potyviridae. Virus Research,* **135**: 213-219.

Were, H.K., Winter, S. and Maiss, E. (2004). Viruses infecting cassava in Kenya. *Plant Disease,* **88**: 17-22.

Westby, A. (2002). Cassava utilization, storage and small-scale processing. In: *Cassava: Biology, Production and Utilization*. Cambridge University Press. CAB International, Wallingford, UK Hillocks, R.J., Thresh, J.M. and Bellotti, A. (eds). Pp281-300.

Wilson, C.R. and Jones, R.A.C. (1992). Resistance to phloem transport of potato leafroll virus in potato plants. *Journal of General Virology*, **73**: 3219-3224.

Winter, S., Koebler, M., Stein, B., Pietruszka, A., Paape, M. and Butgereitt, A. (2010). The analysis of cassava brown streak viruses reveals the presence of a distint virus species causing cassava brown streak disease in East Africa. Journal of General Virology, **91**: 365-376.

Wood, A.P. (1985). A century of development measures and population re-distribution along the Upper Zambezia. In: Population and Development Projects in Africa. Cambridge University Press, Cambridge, UK. Clarke, J.I., Khogali, M. and Kosinski, L.A. (eds.). Pp163-175.

Yadav, J.S., Ogwok, E., Wagaba, H., Basavaprabhu, L., Patil, B.B., Alicai, T., Gaitan-solis, E. Taylor, N.J. and Fauquet, C.M. (2011). RNAi-mediated resistance to *Ugandan Cassava brown streakvirus* in transgenic cassava. *Molecular Plant Pathology* **12**: 677-687.

Zapata, C., Miller, J.C. and Smith, R.H. (1995). An *in vitro* procedure to eradicate *Potato viruses X, Y* and *S* from russet norkotah and two of its strains. *In vitro Cell Development Biology*, **31**: 153-159.

Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G.W., Robinson, D.J., Harrison, B.D. (1997). Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *Journal of General Virology*, **78**: 2101-2111.

Zhou, X., Robinson, D. J. and Harrison, B. D. (1998). Types of variation in DNA-A among isolates of East African cassava mosaic virus from Kenya, Malawi and Tanzania. *Journal of General Virolog,* **79**: 2835-2840.

Zimmermann, A. (1906). Die Krauselkrankheit des Maniok. *Pflanzer,* **2**: 145

## APPENDIX 1

Additional tables and figures for Chapters 4 and 6

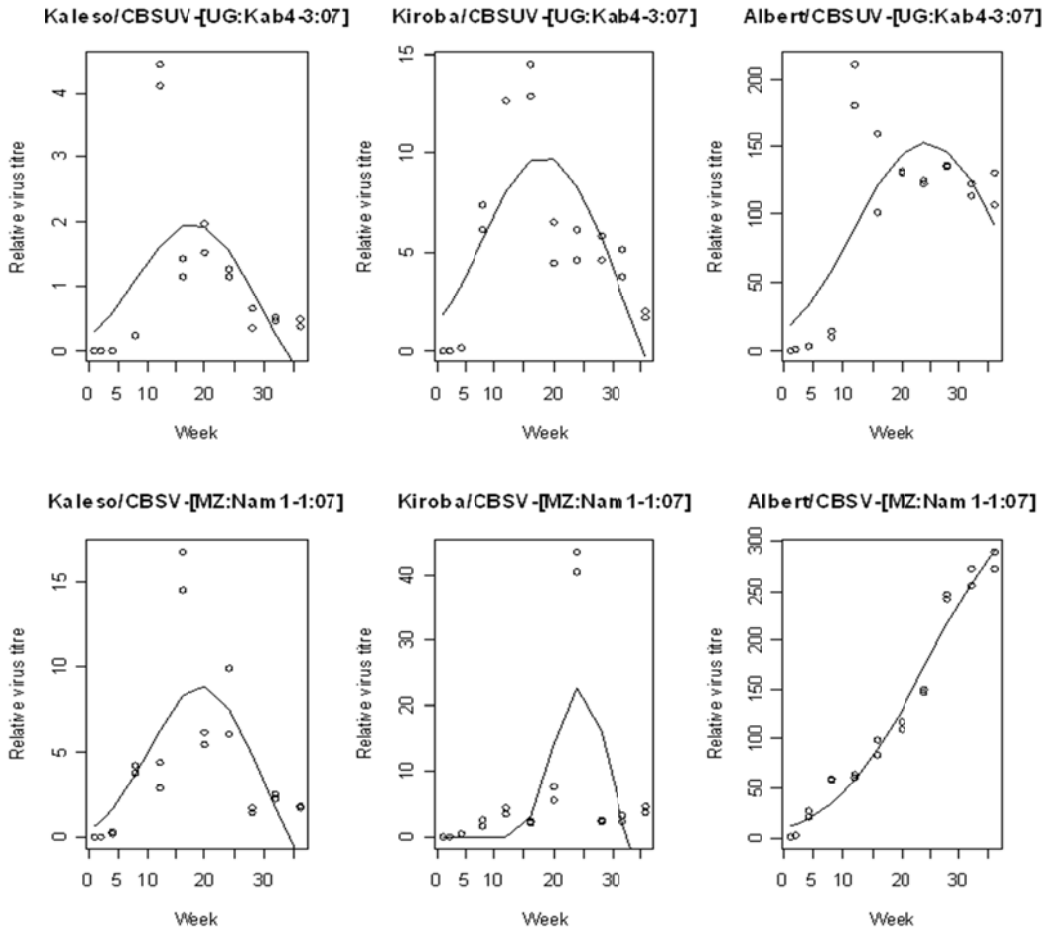
**Appendix 1.1**: Major symptoms expressed by the hebaceous host-plants upon inoculation by the CBSD isolates in the glasshouse

| | UCBSV- | | |
|---|---|---|---|
| Hebaceous host plants | [UG:Kab4-3:07] | [TZ:Kib10-2:03] | [KE:Mwa16-2:08] |
| *Datura stramonium* | LM | LM,LCH | LM,SG |
| *Nicotiana benthamiana* | VC, LM | VC, LM | VC, LM |
| *Nicotiana clevelandii* | MO | LCH | LC, SG |
| *Nicotiana glutinosa* | LCH,LM | LCH/M | LCH,MO, |
| *Nicotiana tabacum* nn | MO,LCH | LCH,M | MO |
| *Nicotiana tabacum* NN | LL | LL | LL |
| *Nicotiana rustica* | LCH,LM | LCH, SG,M | LCH,MO, |
| | CBSV- | | |
| Hebaceous host plants | [TZ:Zan6-2:08] | [MZ:Nam1-1:07] | [TZ:Nal3-1:07] |
| *Datura stramonium* | LM | LM | LM |
| *Nicotiana benthamiana* | VC,LM, | VC,LC,DB | VC,LC,DB |
| *Nicotiana clevelandii* | LCH, SG | LC,S,NEC | LC,S,NEC |
| *Nicotiana glutinosa* | LCH,MO | LCH | MO |
| *Nicotiana tabacum* nn | MO,S | SG | MO |
| *Nicotiana tabacum* NN | LL | LL | LL |
| *Nicotiana rustica* | LCH,LM | LM | SG |

LM = Leaf mottling, VC = Vein clearing, SG = Stunted growth, LC = Leaf collapse, DB = Die back, LCH = Leaf chlorosis, MO = Mosaic, LL = Local lesion

**Appendix 1.2**: Time taken for the first and last experimental host-plant to express symptoms when inoculated by CBSD isolates in the glasshouse

| First/ last symptoms (in weeks) by each CBSD isolate | | | |
|---|---|---|---|
| | UCBSV- | | |
| Herbaceous host plants | [UG:Kab4-3:07] | [TZ:Kib10-2:03] | [KE:Mwa16-2:08] |
| *Datura stramonium* | 1/3 | 2/2 | 2/4 |
| *Nicotiana benthamiana* | 3/5 | 1/4 | 2/3 |
| *Nicotiana clevelandii* | 3/5 | 2/2 | 2/3 |
| *Nicotiana glutinosa* | 1/2 | 3/4 | 3/3 |
| *Nicotiana tabacum* nn | 3/5 | 3/5 | 4/6 |
| *Nicotiana tabacum* NN | 5/7 | 4/6 | 3/5 |
| *Nicotiana rustica* | 3/5 | 2/6 | 2/3 |
| | CBSV- | | |
| Herbaceous host plants | [TZ:Zan6-2:08] | [MZ:Nam1-1:07] | [TZ:Nal3-1:07] |
| *Datura stramonium* | 3/3 | 1/5 | 2/3 |
| *Nicotiana benthamiana* | 1/2 | 3/6 | 3/3 |
| *Nicotiana clevelandii* | 1/3 | 1/2 | 1/2 |
| *Nicotiana glutinosa* | 4/4 | 2/3 | 1/3 |
| *Nicotiana tabacum* nn | 4/5 | 1/8 | 3/3 |
| *Nicotiana tabacum* NN | 3/4 | 1/8 | 3/5 |
| *Nicotiana rustica* | 2/3 | 2/5 | 2/5 |




**Appendix 1.3**: RT-qPCR analysis of UCBSV-[UG:Kab4-3:07] and CBSV-[MZ:Nam1-1:07] titre. The points on the graphs represent data points and the lines join the values predicted by the shifted Gompertz model. The model is fitted to the data using the non-linear least squares method in R. The model was chosen to cope with different Ct levels for the different virus variety combinations as described by Cowley (2009).

**Appendix 1.4**: Relationship between visual observations and RT-PCR for CBSV detection in vector transmission experiments

| Experiment 1 | Visual observations[a] | | | RT-PCR detection[b] | | |
|---|---|---|---|---|---|---|
| Serial number of plants | Albert | Kiroba | Kaleso | Albert | Kiroba | Kaleso |
| 1 | + | + | - | + | + | - |
| 2 | - | - | - | - | - | - |
| 3 | + | + | - | + | + | - |
| 4 | + | + | - | + | + | - |
| 5 | - | - | + | - | - | + |
| 6 | - | - | - | - | - | - |
| 7 | - | + | - | - | + | - |
| 8 | + | + | - | + | + | - |
| 9 | + | - | - | + | - | - |
| 10 | + | + | - | + | + | - |
| Healthy control[c] | - | - | - | - | - | - |
| Transmission rate (%)[d] | 60 | 60 | 10 | 60 | 60 | 10 |

[a]CBSV was visually observed and plants were considered positive or negative for CBSV based on typical CBSD symptoms on leaves.
[b]Plants were considered positive for CBSV only when the bands of the expected sizes were generated by RT-PCR.
[d]Percent transmission in each variety in the experiment 1. - and + indicate negative and positive in RT-PCR, respectively.

**Appendix 1.5**: Relationship between visual observations and RT-PCR for CBSV detection in vector transmission experiments

| Experiment 2 | Visual observations[a] | | | RT-PCR detection[b] | | |
|---|---|---|---|---|---|---|
| Serial number of plants | Albert | Kiroba | Kaleso | Albert | Kiroba | Kaleso |
| 1 | + | + | - | + | + | - |
| 2 | - | + | - | - | + | - |
| 3 | + | + | - | + | + | - |
| 4 | - | - | - | - | + | - |
| 5 | - | - | - | - | - | - |
| 6 | + | - | + | + | - | - |
| 7 | - | - | - | - | - | - |
| 8 | + | + | - | + | + | - |
| 9 | + | - | - | + | - | - |
| 10 | + | - | - | + | - | - |
| Healthy control[c] | - | - | - | - | - | - |
| Transmission rate (%)[d] | 60 | 40 | 10 | 60 | 50 | 0 |

[a]CBSV was visually observed and plants were considered positive or negative for CBSV based on typical CBSD symptoms on leaves.
[b]Plants were considered positive for CBSV only when the bands of the expected sizes were generated by RT-PCR.
[d]Percent transmission in each variety in the experiment 2. - and + indicate negative and positive in RT-PCR, respectively.

**Appendix 1.6**: Relationship between visual observations and RT-PCR for CBSV detection in vector transmission experiments

| Experiment 3 | Visual observations[a] | | | RT-PCR detection[b] | | |
|---|---|---|---|---|---|---|
| Serial number of plants | Albert | Kiroba | Kaleso | Albert | Kiroba | Kaleso |
| 1 | - | - | - | - | - | - |
| 2 | - | - | - | - | - | - |
| 3 | + | - | - | + | - | - |
| 4 | - | - | - | - | - | - |
| 5 | + | + | - | + | + | - |
| 6 | - | + | - | - | + | - |
| 7 | - | - | - | | + | - |
| 8 | + | - | - | + | - | - |
| 9 | + | - | - | + | - | - |
| 10 | + | - | - | + | - | - |
| Healthy control[c] | - | - | - | - | - | - |
| Transmission rate (%)[d] | 50 | 20 | 0 | 50 | 30 | 0 |

[a]CBSV was visually observed and plants were considered positive or negative for CBSV based on typical CBSD symptoms on leaves.
[b]Plants were considered positive for CBSV only when the bands of the expected sizes were generated by RT-PCR.
[d]Percent transmission in each variety in the experiment 3. - and + indicate negative and positive in RT-PCR, respectively.

## APPENDIX 2

Statistical Analysis of Data

**Appendix 2.1**: Summary of two-way analysis of variance (ANOVA) for CBSD symptoms severity on cassava varieties

| parameter | d.f | s.s | m.s.s. | F- value | P- value |
|---|---|---|---|---|---|
| Variety (v) | 4 | 30.4 | 7.6 | 39.7 | P< 0.001 |
| Isolate (I) | 5 | 155.9 | 31.2 | 163.1 | P< 0.001 |
| V x I | 20 | 9.8 | 0.5 | 2.6 | P< 0.003 |

d.f = degree of freedom, P = probability at 95% confidence level

**Appendix 2.2**: Summary of analysis of deviance (Chi-square) for the significant effects of Varieties, isolates and their effects on infected cuttings sprouting

| effect | d.f | ($X^2$) | P-value |
|---|---|---|---|
| Variety (V) | 2 | 21.8 | P<0.0001 |
| Isolate (I) | 1 | 13.3 | P<0.0002 |
| V x I | 2 | 0.4 | NS |

d.f = degree of freedom, $X^2$ = chi-square, N.S = no significant differences

**Appendix 2.3**: Summary of analysis of deviance (Chi-square) for the significant effects of Varieties, isolates and their effects on CBSD revrsion in cassava varieties

| effect | d.f | ($X^2$) | P-value |
|---|---|---|---|
| Variety (V) | 2 | 15.6 | P<0.0004 |
| Isolate (I) | 1 | 2.9 | NS |
| V x I | 2 | 0.4 | NS |

d.f = degree of freedom, $X^2$ = chi-square, N.S = no significant differences

**Appendix 2.4**: Summary of two-way analysis of variance (ANOVA) for the effects of cutting position on CBSD reversion.

| parameter | d.f | s.s | m.s.s. | F- value | P- value |
|---|---|---|---|---|---|
| Variety (v) | 2 | 0.6 | 0.3 | 13.5 | P< 0.0001 |
| Isolate (I) | 1 | 0.2 | 0.2 | 7.5 | P< 0.0093 |
| Cutting position (CP) | 2 | 0.2 | 0.1 | 5.2 | P< 0.0100 |
| V x I | 2 | 0.1 | 0.0 | 1.6 | NS |
| V x CP | 4 | 0.0 | 0.0 | 0.4 | NS |
| I x CP | 2 | 0.0 | 0.0 | 0.0 | NS |
| V x I x CP | 4 | 0.0 | 0.0 | 0.01 | NS |

d.f = degree of freedom, s.s= sum of square, m.s.s = mean sum of square, N.S = no significant differences, P = probability at 95% confidence level.

**Appendix 2.5**: Summary of two-way analysis of variance (ANOVA) on the effects of cassava varieties and fecundity of *B. tabaci.*

| parameter | d.f | s.s | m.s.s. | F -Value | P-Value |
|---|---|---|---|---|---|
| Eggs layed on varieties | 2 | 1163.0 | 582.0 | 0.1 | N.S |
| Nymphs on varieties | 2 | 1422.0 | 711.0 | 0.1 | N.S |
| Adults eclosion on varieties | 2 | 685.0 | 342.0 | 0.1 | N.S |
| Nymphs x adults x variety | 2 | 7.2 | 3.58 | 0.3 | N.S |

d.f: Degree of freedom, s.s= sum of square, m.s.s = mean sum of square, N.S = no significant differences P: probability at 95% confidence level.

## APPENDIX 3

List of outputs generated from this and other related research on CBSD as follows:

1) **I. U. Mohammed**, M. M. Abarshi, B. Muli, R. J. Hillocks, and M. N. Maruthi (2011). The symptom and genetic diversity of cassava brown streak viruses. *Advances in Virolog,* **10**:1155-1165.

2) Abarshi, M. M., **Mohammed, I. U**., Legg, J. P., Kumar, L., Hillocks, R. J. and Maruthi, M. N. (2012). Multiplex RT-PCR assays for the simultaneous detection of both RNA and DNA viruses infecting cassava and the common occurrence of mixed infections by two cassava brown streak viruses in East Africa. *Journal of Virological Methods,* **18**: 176-179.

3) M. M. Abarshi, **I. U. Mohammed**, P. Wasswa, R. J. Hillocks, J. Holt, J. P. Legg, S. E. Seal and M. N. Maruthi, (2010). Optimization of diagnostic RT-PCR protocols and sampling procedures for the reliable and cost effective detection of Cassava brown streak virus. *Journal of Virological Methods*, **163**: 353-359.

4) Patil, B.L., Ogwok, E., Wagaba, H., Yadav, J.S., Bagewadi, B., Taylor, N.J., Kreuze, J.F., Maruthi, M.N., **Mohammed, I.U**., Alicai, T. and Fauquet, C.M. (2010). RNAi mediated resistance to diverse isolates belonging to two virus species involved in cassava brown streak disease. *Molecular Plant Pathology* **12**: 31-41.

### Abstracts and poster presented in international conferences

**Mohammed, I. U**., Abarshi, M. M., Hillocks R. J. and Maruthi, M. N. (2012). Mechanisms of resistance to Cassava brown streak disease in cassava varieties. Oral presentation in: the conference 'Advances in Plant Virology' 28-30th March 2012 in Dublin, North Ireland.

**Mohammed, I. U**., Abarshi, M. M., Hillocks R. J. and Maruthi, M. N. (2012). Developing methods to eliminate UCBSV and CBSV from infected cassava varieties. Poster in: the conference 'Advances in Plant Virology' 28-30th March 2012 in Dublin, North Ireland.

**Mohammed, I. U**., Abarshi, M. M., Muli B., Hillocks R. J. and Maruthi, M. N. (2010). Virus-host interaction studies reveal the occurrence of virulent and milder forms of cassava brown streak virus. Oral presentation in: the conference 'Advances in Plant Virology' 5-7 September 2010 in Netherlands.

**Mohammed, I. U**., Abarshi, M. M., Muli B., Hillocks R. J. and Maruthi, M. N. (2010). Virus-host interactions in cassava brown streak disease pathosystem. Poster in: the conference 'Advances in Plant Virology' 5-7 September 2010 in Netherlands.

Maruthi, M.N. Jeremiah, S., **Mohammed, I.U**. and Legg, J.P. (2011). Investigations on Cassava brown streak virus transmission by the whitefly, Bemisia tabaci. The Fourth European Whitefly Symposium. Held at Rehovot, Israel, 11-16 September, 2011.

Maruthi, M.N, Jeremiah, S., **Mohammed, I.U**., Kumar, L. and Legg, J.P. (2010). Investigations on Cassava brown streak virus transmission by whiteflies, International Workshop on CBSD, May 2010, Uganda.

Maruthi, M.N, Abarshi, M. M., **Mohammed, I. U**., Seal, S. E. Hillocks, R. J., Kumar, L. and Legg, J.P. (2010). Cassava brown streak virus diversity and development of improved virus diagnostics. In: Plant viruses: Exploiting agricultural and Natural ecosystems. 11th International plant virus epidemiology symposium and 3rd Workshop of the plant virus ecology network. Held at Cornell, University Ithaca, New York, USA. 20 – 24 June, 2010. 17p.