QUANTIFICATION OF CASSAVA MOSAIC GEMINIVIRUSES AND CASSAVA BROWN STREAK VIRUSES

By

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DEDICATION

This work is dedicated to my mother, Hellena Sikwese, who took extraordinary courage to raise and educate me after the death of my father when I was just a little boy and to my wife, Evelyn, for enduring my absence in course of my studies.

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LIST OF ABBREVIATIONS

$\Delta\Delta$CT	Delta-delta Ct
ΔRn	Delta Rn
°N	North latitude
°S	South latitude
ACMV	African cassava mosaic virus
ANOVA	Analysis of variance
bp	Base pair(s)
$CaCl_2$	Calsium Chloride
CBBD	Cassava bacterial blight disease
CBSD	Cassava brown streak disease
CBSV	Cassava brown streak virus
CGM	Cassava green mite
CM	Cassava mealy bug
CMD	Cassava mosaic disease
CMG	Cassava mosaic geminivirus
CMMGV	Cassava mosaic Madagascar virus
COX	Cytochrome oxidase
CRD	Completely randomized design
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
EACMCV	East African cassava mosaic Cameroon virus
EACMMV	East African cassava mosaic Malawi virus
EACMV (UgA)	East African cassava mosaic virus - Uganda variant
EACMZV	East African cassava mosaic Zanzibar virus
EDTA	Ethylenediaminetetraacetic acid
F1	First filial generation
FAO	Food and Agricultural Organisation
ICMV	Indian cassava mosaic virus
IITA	International Institute of Tropical Agriculture
LSD	Least significant difference
MCMV	Madagascar cassava mosaic virus
MAP	Months after planting
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride

OP	Open pollinated
PCR	Polymerase chain reaction
pH	Power of hydrogen
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcription PCR
RCBD	Randomised complete block design
RNA	Ribose nucleic acid
RQ	Relative quantity
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
RuBisCO	Ribulose-1,5- bisphosphate carboxylase/ oxygenase
SACMV	South African cassava mosaic virus
SDS	Sodium dodecyl sulphate
SLCMV	Sri Lankan cassava mosaic virus
Ssp	Subspecies
ssRNA	Single stranded RNA
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TNA	Total nucleic acid
UCBSV	Ugandan cassava brown streak virus
v/v	Volume per volume
w/v	Weight per volume

LIST OF SI UNITS

°C	Degrees Celsius
cm	Centimetre(s)
g	Gram(s)
h	Hour(s)
ha	Hectare(s)
m	Metre(s)
M	Molar(s)
min	Minute(s)
mg	Miligram(s)
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar(s)
ng	Nanogram
sec	Second(s)
U	Unit(s)
µl	Microlitre(s)
µm	Micrometre(s)
µM	Micromolar(s)

CHAPTER 1
GENERAL INTRODUCTION

1.1 Motivation and objectives

Cassava (*Manihot esculenta* Crantz) is a perennial woody plant (Onwueme, 1978). It is cultivated in Africa, Asia and Latin America. Due to its tolerance to drought, cassava cultivation has expanded into marginal environments, particularly in regions with poor soils and lengthy dry seasons, hence it is regarded as a food security crop (El-Sharkawy, 1993).

The mature cassava plant has a woody stem which is cylindrical in shape and is formed by alternating nodes and internodes. On the nodes of the oldest parts of the stem, there are protuberances, which are the scars left by the plant's first leaves. A plant grown from stem cuttings can produce as many primary stems as there are viable buds on the cutting, but in some cultivars with strong apical dominance, only one stem develops (Alves, 2002). Though cassava is propagated mainly using stem cuttings simply called stakes, propagation by seeds is done in plant breeding (Onwueme, 1978). Scientists, specifically plant breeders, use sexual seeds for breeding trials due to the heterotic nature of cassava. Production and viability of seed differ, mainly due to the quality of the female parent (Kawano, 1980). One viable seed per fruit is normally achieved in controlled pollinations, but there is the possibility of getting a maximum of three in the trilocular ovary (Jennings, 1963).

Cassava is a source of dietary energy in sub-Saharan Africa (Scott et al., 2000) as the storage roots are rich in carbohydrates (>85%), though poor in protein (2-3%, dry weight basis) (Hahn, 1989). The leaves are consumed as vegetable in Africa and they are a good source of proteins and some vitamins (Moyo et al., 1998). Cassava is the most important root crop in Malawi and is grown across the country as a staple food crop for more than 30% of the people along the central and northern lake shore areas of Lake Malawi and the Shire highlands (Moyo et al., 1998; Alene et al., 2013). Cassava is becoming an important industrial crop (Benesi, 2002; Benesi et al., 2004). Cassava starch is used as a raw material for further processing in the production of paper, textiles and monosodium glutamate, an important flavouring agent in Asian cooking (Benesi, 2002).

Although cassava is important, there are a number of production constraints of which some include the two viral diseases namely cassava mosaic disease (CMD) and cassava brown streak disease (CBSD). CMD mostly attacks leaves and CBSD attacks leaves, stems and

roots but it has the largest effect on the roots. The two diseases are known to be transmitted by whiteflies, *Bemisia tabaci* (Maruthi et al., 2005). Benesi (2005) reported that farmers in northern Malawi mistook CMD symptoms as the effects of the onset of the rainy season. The following prevailing virus strains are known to cause CMD in Africa: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Zanzibar virus* (EACMZV), Ugandan variant of EACMV (EACMV-Ug), *South African cassava mosaic virus* (SACMV) and *cassava mosaic Madagascar virus* (CMMGV) (Malathi et al., 1987; Geddes, 1990; Hong et al., 1993; Zhou et al., 1997; Thresh et al., 1998; Rey et al., 2012). *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) are the two virus strains known to cause CBSD (Mbanzibwa et al., 2009). In Malawi CMD is caused by *East African cassava mosaic Malawi virus* (EACMMV) and SACMV (Alabi et al., 2011; Aloyce et al., 2013). Both CBSV and UCBSV are prevalent in Malawi (Mbewe et al., 2014).

CMD and CBSD are of great concern in Malawi (I.R.M. Benesi, personal communication). A number of strategies have been employed to counter this problem, which include breeding by making crosses and introduction of botanical seeds from other places and regions. However the safety of this strategy to the recipient country or place in terms of the potential of disease transfer from plant to seed and then from seed to seedling, is to be thoroughly verified. Since cassava is extremely heterogeneous, there is the possibility of developing different traits through crossing. If these viral diseases are not transmitted through seeds, scientists can produce virus free seeds. This will be another mechanism of ensuring that clean seeds and more planting materials are available for propagation and will help in the fight against CMD and CBSD.

Genetic recombination can generate progeny resistant to CMD and CBSD. Responses of crops to pathogens are dependent on genetic aspects of the crop, among other factors. In breeding programmes, breeders look for traits of interest based on the objectives of the breeding programme. To make the fight against CMD and CBSD more efficient, plant breeders need to look for traits consistent with resistance or tolerance to the two diseases. Many studies have focussed on the severity of the diseases; however, many of the severity studies that have been done thus far deal mainly with visual symptoms in the field. The molecular aspects of the response of various genotypes to cassava mosaic geminivirus (CMG) and CBSV has not been investigated in depth, and in Malawi no research has been reported in terms of viral quantities, hence will be of importance in this regard. Knowledge on the rate of accumulation of the viruses in particular cassava families or genotypes during their growth cycle in the field, will help to develop a disease control strategy for the viral

diseases of interest. Viral load quantification will help to investigate the correlation between CMD and CBSD symptom severity and CMG and CBSV accumulation in genotypes of cassava. This information will help to check which families or genotypes have no or low quantities of viruses which is essential in breeding programmes to decide which families or genotypes to select for further evaluation in order to deal with these diseases.

Most of the CMG and CBSV diagnoses using polymerase chain reaction (PCR) are done using DNA and RNA respectively, extracted from cassava leaves. During certain times of the year, cassava plants undergo senescence in which plants shed their leaves, which makes it almost impossible to do disease severity studies using visual inspection, as well as sampling for laboratory diagnosis. However, although leaves might be the tissue that is mostly used for DNA/RNA extraction, other cassava tissues might be of equal importance. The possible use of tissues such as the stem cortex, root parenchyma and root cortex for laboratory diagnosis can be validated once their viral quantities are determined relative to those of leaves. Therefore, there is a need to determine variations in virus quantities in different cassava organs and tissues. This will help in the diagnosis of CMG and CBSV even when some parts of a plant are missing.

Nucleic acids based technology has been employed in virus detection and quantification. This has been possible with the development of PCR based technology. Virus quantities may vary depending on genotype, environment and stage of crop growth (Busogoro et al., 2008; Tadeo, 2014). There are a number of methods used for virus quantification, which include method of absolute quantification using real-time PCR and another method measuring relative quantities. In absolute quantification the aim is to find the actual virus quantities while in relative quantification, the virus quantities are based on comparison with the other samples (Moreno et al., 2011; Adams et al., 2013).

The aim of this research was to quantify cassava viruses and improve disease diagnosis. Under the aim, there were three objectives which are:
- (a) To quantify CBSV, UCBSV, SACMV and EACMMV in cassava F1 progeny seedlings in the field.
- (b) To determine variations in quantities of CBSV, UCBSV, SACMV and EACMMV in different cassava tissues of three cassava genotypes in Malawi.
- (c) To detect the presence of CBSV, UCBSV, SACMV and EACMMV in seedlings grown from seeds obtained from diseased plants.

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CHAPTER 2
LITERATURE REVIEW

2.1 Cassava taxonomy

Cassava (*Manihot esculenta* Crantz) is a monoecious plant. It is diploid (2n = 36), eudicot and belongs to the family *Euphorbiaceae* (De Carvalho and Guerra, 2002; Mbanzibwa et al., 2011) but of all the *Euphorbiaceae* it is only *Manihot esculenta* that produces tuberous roots, and this has led to it being domesticated (Chiwona-Karltun, 2001).

Plants in the family *Euphorbiaceae* are characterised by vessels composed of sector cells and include commercially important crops such as rubber trees (*Hevea brasiliensis* Müll), oil plants (*Ricinus comunis* Linnaeus), ornamental plants (Euphorbiaceae species) and root crops (Osiru et al., 1996; Chiwona-Karltun, 2001).

2.2 Origin of cassava

Earlier, it was assumed that cassava has no known ancestry (Allem, 2001). The genus *Manihot* has at least 100 species but *M. esculenta* Crantz is the only cultivated species (Nassar and Ortiz, 2007). Cassava, *M. esculenta* subsp. *esculenta* that is currently cultivated originated from wild subspecies *M. esculenta* subsp. *flabellifolia*. It was domesticated from populations of *M. esculenta* subsp. *flabellifolia* that occur along the southern rim of the Amazon basin. The progenitor of cassava is restricted to parts of the South American neotropical mainland. However, Allem (2001) suggested that there might be a possibility that the cassava ancestor evolved in the Brazilian Cerrado before reaching the Amazon. In Africa, it was brought in by the Portuguese. It was grown with the main purpose of providing chips to slaves (Ross, 1975; Cock et al., 1985). It is believed to have been introduced into Malawi between the 17th and 19th centuries (Sauti, 1981).

2.3 Cassava morphology

In many cases, cassava cultivars are distinguished from each other on the basis of their morphology (Onwueme, 1978). A cassava plant, like many other plants, is made up of organs such as stems, leaves, roots and flowers.

2.3.1 Stems

The stem produces secondary branches that produce other successive branching. Alves (2002) described this as di-, tri- or tetra-chotomous division. These branchings, which are induced by flowering, have been called 'reproductive branchings'. Stem morphological and

agronomic characteristics are very important in characterising a cultivar. The variation of these characteristics depends on cultivar, cultural practices and climatic conditions. In the primary state the stem is surrounded by an epidermis beneath which is the cortex. Internal to the cortex are vascular bundles. The bundles contain phloem and xylem. At the centre there is the pith which is composed of parenchyma cells. The stem becomes woodier as the plant grows (Onwueme, 1978).

2.3.2 Leaves

The leaves of cassava are arranged spirally on raised nodal portions on the stem. Each leaf is subtended by three to five stipules. The leaf petiole varies from 5-30 cm in length (Onwueme, 1978). The lower mesophyll surface of cassava leaf has papillose-type epidermal cells but the upper surface is smooth, with some scattered stomata and trichomes (Angelov et al., 1993). Leaves of cassava have green bundle sheath cells, with small, thin walled cells, which are separated below the palisade cells; these cells transport the photosynthates apart from performing photosynthesis (Alves, 2002). The leaf canopy of cassava affects the growth and productivity as it is able to intercept solar radiation during the growth cycle of the plant. The canopy influences the photosynthetic potential and performance of leaves under prevailing environmental conditions (De Tafur et al., 1997; El-Sharkawy, 2004).

2.3.3 Roots

The roots are the main storage organs in cassava. The tuberous roots result from the thickening of fibrous roots (Gbagedesin et al., 2013); thus the soil in which the crop grows is penetrated by small, thin roots, and growth and thickening begins only after penetration of the roots. Alves (2002) reported that anatomically, cassava roots are not tubers, but true roots that cannot be used for vegetative propagation. While some roots thicken, the other fibrous roots remain thin and keep on functioning in the absorption of water and nutrients (Alves, 2002).

In cassava plants propagated from true seeds, a primary tap root system develops (Alves, 2002); unlike plants propagated from cuttings. The development of storage roots depends on the genotype and photo sensitivity of the plants (Onwueme, 1978; Were, 2011). Under short day conditions tuberisation is high, while under long day conditions tuberisation is delayed (Onwueme, 1978); as such cassava is regarded as a short day plant (Hunt et al., 1977).

The mature cassava storage root has three distinct tissues. These are the periderm, cortex and parenchyma (Onwueme, 1978; Alves, 2002). The parenchyma is the edible part of the root and is known to contribute about 85% of total weight of the root (Wheatley and Chuzel, 1993). The parenchyma consists of xylem vessels (Wheatley and Chuzel, 1993). The peel layer contains the sclerenchyma, cortical parenchyma and phloem, and it contributes approximately 10–20% of root weight (Onwueme, 1978). As indicated in Figure 2.1 the periderm, which contributes about 3% of total root weight, is the outermost thin layer and it is usually sloughed off as it grows.
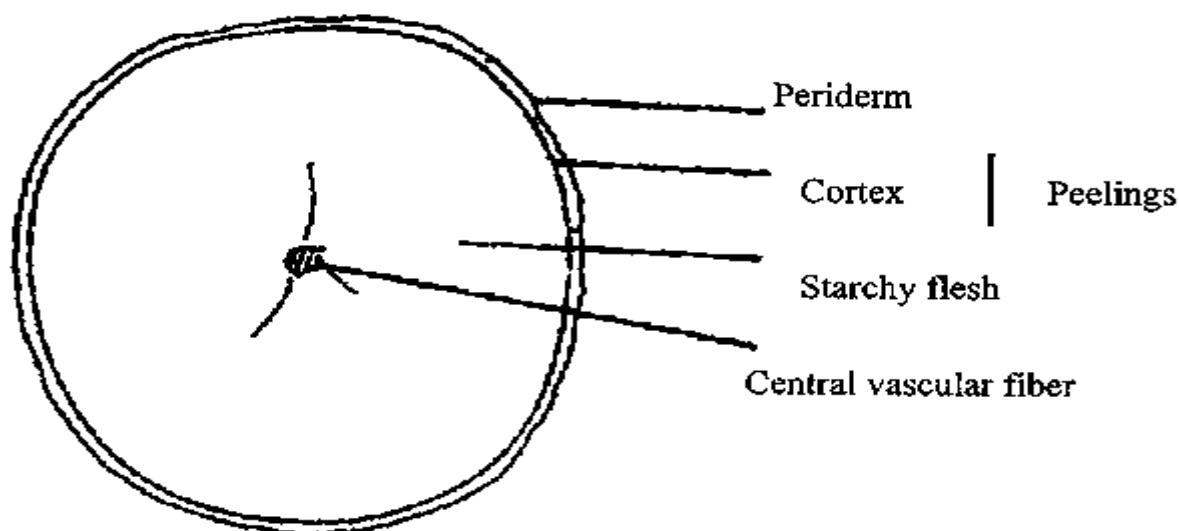
Figure 2.1 Parts of a cassava root (cross sectional view) (www.fao.org, 26 July, 2015)

The size and shape of the root depend on the genotype and environmental conditions; however, variability in size within a genotype is usually greater in cassava compared to other root crops (Wheatley and Chuzel, 1993).

The xylem tissue and schlerenchymous fibres are composed of lignin, a hard variable material of cross-linked phenylpropane units, which adds stiffness to the cell walls (Buschmann et al., 2000). The outer layers of the cassava tuber constitute the periderm: a tissue that replaces the epidermis in most stems and roots having secondary growth. The periderm is made up of an outer layer of cork tissue and an inner layer of living parenchyma cells (Buschmann, 2000).

Some researchers found differences in anatomical structures between roots of cultivated and wild species. For example anatomical differences among *Manihot* species and

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varieties were found in the epidermal and exodermal cell shape and wall thickness, content of cortical parenchyma and number of xylem poles (Bomfim et al., 2011). Wall thickness of the epidermis and exodermis of the tap root are similar in all species, while in the lateral root there are differences in cell shape and wall thickness. Epidermal cells with thick walls were found in the tap root of all species and in lateral roots of cassava varieties. The variation in the number of xylem poles of cassava varieties has been reported to be larger (4-8) than in wild species (4-6), and appears to support the hybrid origin of cassava (Bomfim et al., 2011).

2.4 Seed and reproduction biology

Fertilised cassava seed is viable approximately two months after pollination and the fruit matures about three months after pollination (Ceballos et al., 2002); this means that it takes 3 to 5 months to mature (Onwueme, 1978). The cassava plant produces fruits which are trilocular schizocarp from which true seeds are produced, and the seeds are approximately 10 mm long and 4 to 6 mm thick (Alves, 2002). Ceballos et al. (2004) indicated that from each flower that has been pollinated manually, one to two viable seeds can be obtained.

Seed dormancy exists in cassava. Seeds that have been newly harvested are dormant, and require about 3 to 6 months of storage at room temperature before they are planted (Jennings and Iglesias, 2002). Temperature is one of the factors that affect seed germination. Cassava seeds germinate well in conditions of low humidity and complete darkness. High temperatures (35°C) promote seed germination, while lower temperatures (25°C) reduce germination (Pujol et al., 2002).

Cassava is very heterogeneous; therefore using sexual seeds for propagation gives rise to a wide diversity of phenotypes, which is of interest to breeders, but propagation by sexual seeds is not easy (Ceballos et al., 2004). The seeds are ellipsoidal and are 1 to 1.5 cm in length (Onwueme, 1978). Heterozygous volunteers which result from natural outcrossing are larger and more vigorous than inbred seedlings which were affected by inbreeding depression; as such they are preferred and retained (Kawano, 1980). Seedlings from sexual seeds are initially smaller and weaker than plants developed from stakes and need to be delicately handled for them to be established. Therefore, cassava is usually propagated by farmers and other researchers using stem cuttings.

Cassava has large sized pollen grains which are sticky and adhere to insect bodies and this facilitates cross pollination; as such wind pollination has little effect (Kawano, 1980; Chavarriaga-Aguirre and Halsey, 2005). In Colombia and Africa respectively, it is pollinated mainly by several species of wasp (*Polistes* spp.) and honeybees (*Apis mellifera*)

(Kawano, 1980). The opening of male and female flowers at the same time on different branches of plants belonging to the same genotype can result in self-pollination (Jennings and Iglesias, 2002).

Temperature affects the pollen shed in flowers and germination in many plants. Extreme temperatures may result in inadequate amounts of pollen which may impede artificial pollination. Pollen quality and quantity are also influenced by the relative humidity of the prevailing environment in which plants are cultivated (Acquaah, 2007). Larger pollen grains have better germination (about 60%) under *in vitro* conditions (40°C temperature for 2 hrs) than the smaller ones whose viability may be less than 20% (Chavarriaga-Aguirre and Halsey, 2005). In breeding programmes, it is important to have a good knowledge of the duration for which stigma of the flower and pollen from the anther remain receptive and viable respectively. Since it does not take long for pollen to lose viability, breeders usually try to reduce the time of the crossing by pollinating the stigmas within one hour after collection of pollen for successful fertilisation (Chavarriaga-Aguirre and Halsey, 2005).

In breeding blocks or seed multiplication fields, netting bags are usually placed around the fruit after pollination, to trap the dehiscing seeds from the mature fruit. On average between one to two seeds, maximum three, are obtained per cross using the above technique (Kawano, 1980; Ceballos et al., 2004).

2.5 Floral biology

Cassava is a monoecious flowering plant that bears male and female flowers on the same inflorescence on the same plant (Ceballos et al., 2004). The cassava plant bears its flowers on terminal panicles and the branch axis is continuous with the axis of the panicle inflorescence (Onwueme, 1978). The stigma and anthers occur in different flowers on the same plant (Kawano, 1980). The female flowers are slightly larger than male flowers which can be about 0.5 cm in diameter (Chavarriaga-Aguirre and Halsey, 2005).

Cassava flowers remain open for about a day after opening at around mid-day (Ceballos et al., 2002). On a particular branch, as mentioned above, female flowers are the first to open; the male flowers open later (about one to two weeks after the female flowers), a characteristic called protogyny (Chavarriaga-Aguirre and Halsey, 2005). However, Alves (2002) indicated that male and female flowers on different branches can open simultaneously. Early opening of females facilitates out crossing by insects. When plants flower, self- and sib-fertilisation may take place depending on the genotype used, the

presence of pollinators and the environment in which plants grow (Kawano, 1980; Jennings and Iglesias, 2002).

Flowering is usually influenced by environmental factors such as photoperiod and temperature. In a particular environment a genotype may produce flowers, but fail to do so in another or may produce, but abort flowers (Tang et al., 1983). Genotypes that do not flower in warm, low altitude zones may flower in cooler, high altitude zones (Kawano, 2003). For the purposes of breeding, cassava genotypes are classified into different regions just like many other crops, so that breeders/researchers can make use of the knowledge of the flowering habits of the plants to be crossed (Ceballos et al., 2002). Usually, cassava produces more male flowers than female flowers per branching (Kawano, 1980; Nunekpeku et al., 2013).

Photoperiod is one aspect that affects floral induction in many plants. In some genotypes, induction of flowering depends on photoperiods of up to 16-hour day length; associated with an optimum temperature of 24ºC (Keating, 1982; Tang et al., 1983; Alves, 2002). Flowering has been observed to be promoted by spraying growth hormones, indolacetic acid, naphthalene acetic acid and ascorbic acid on leaves. A longer photoperiod may reduce the rate of flower abortion (Tang et al., 1983).

Flowers play a pivotal role in hybridisation of plants. Although cassava is known to be drought tolerant, long dry weather spells inhibit flowering (Kawano, 1980). For a breeding programme to be successful, the flowers need to be in overall good health and be mature enough to receive pollen from the male. However, the technique employed for crossing varies, depending on the floral biology. Floral biology refers to time at which pollen is shed, flower shape and size; it also refers to whether the flower is complete or incomplete or self- or cross-pollinated (Acquaah, 2007).

2.6 Propagation
2.6.1 Propagation using cuttings
Cassava can either be propagated using stem cuttings commonly known as stakes or by seed, but propagation by stakes is the most common method used (Alves, 2002). Cuttings are made from a single mother plant at the age of 8 to 18 months for propagation. Usually farmers in Malawi use stakes from the previous crop, but sometimes they get the planting materials from research stations. The stakes (about 15 to 30 cm) are planted either vertically, horizontally or inclined on ridges (El-Sharkawy, 2004). Cassava planted from cuttings usually has good establishment and they are stronger than those planted from

seeds (Osiru et al., 1996; Nassar and Ortiz, 2007). However, the use of stakes from previous crops for propagation is an easy way of transmitting diseases and results in accumulation of viruses in cassava fields. As such the use of true seeds might be of some importance.

2.6.2 Propagation using true/sexual seeds

Sexual seeds are used mostly by plant breeders (Onwueme, 1978). At research stations in Malawi, sexual seed is produced for creating new genetic variation in breeding programmes through controlled or uncontrolled pollination. Using sexual seeds to propagate cassava result in plants that are genetically diverse; therefore sexual seeds are used to generate new varieties (Onwueme, 1978; Alves, 2002).

2.7 Importance of cassava

Cassava is among the most important sources of dietary energy in sub-Saharan Africa (Scott et al., 2000) and is drought tolerant (Benesi, 2005). In terms of the amount of calories consumed, cassava is Africa's second most important staple food after maize (Nweke, 2004).

When leaves are consumed as a vegetable, cassava provides proteins, lipids, vitamins and minerals such as calcium and iron (Montagnac et al., 2009). Roots are used to produce starch which is used in the food industry. The starch is also used in glass, mineral wool and clay as an adhesive. In some countries, cassava is used for production of ethanol for various purposes of which one is transport fuel. Cassava also acts as a base in alcoholic beverages. Roots are processed into products such as flour, chips and starch which are easily stored (Onwueme, 1978). Animals such as cattle, goats and sheep can be fed on fresh tubers (Onwueme, 1978; El-Sharkawy, 2006).

At least 30% of the people living along the central and northern lake shore areas of Lake Malawi and the Shire highlands regard cassava as their staple food (Benesi, 2005; Alene et al., 2013). It is eaten raw as a snack, boiled or roasted (Sauti et al., 1994; Moyo et al., 1998). It is also eaten as mash or fried. The cassava flour is used to prepare "kondowole" in some parts of the country.

2.8 Cassava production in Malawi

More than 50% of world's cassava production occurs in sub-Saharan Africa (FAO, 2014). Since it is able to grow in various agro-ecological zones (Akinbo et al., 2012), it is cultivated

throughout Malawi, but especially in the lake shore areas and Shire highlands (Benesi, 2005; Alene et al., 2013). Cassava production has increased drastically over the years (Rusike et al., 2010) which gives hope that the production will continue increasing, taking into consideration the efforts of the Malawi Government and other projects dealing with cassava breeding and production. Cassava is mainly produced by smallholder farmers. Farmers have been able to realise 8-39 ton ha^{-1} yield with an average of 19 ton ha^{-1} in some districts of the country, depending on the climatic and soil conditions (Schöning and Mkumbira, 2007).

2.9 Production constraints of cassava

Cassava production is limited by both biotic and abiotic factors (Adjata et al., 2011; Gbadegesin et al., 2013). The biotic factors include pests and diseases. Common pests of cassava include cassava mealybug (CM), cassava green mite (CGM) and variegated grasshopper. Diseases that affect cassava production include CMD, CBSD and cassava bacterial blight disease (CBBD) (Gbadegesin et al., 2013). Among the important diseases of cassava are CMD and CBSD. Cassava is faced with a number of other constraints of which some are the shortage of good quality varieties that are high yielding, problems with storage leading to post-harvest losses, the use of inappropriate cultural practices, and inadequate access to clean and healthy planting materials that are virus free (Sauti et al., 1994; Benesi et al., 2003).

Since the use of sexual seeds is not viable in cassava production, the use of vegetative materials is most applicable, but this leads to build up of virus diseases (Calvert and Thresh, 2002). The production therefore will depend on the supply of good quality and healthy stakes. However, the rate of multiplication of the materials (cuttings) used in vegetative propagation is very low compared to the use of sexual seeds. Furthermore, stem cuttings are usually very bulky and highly perishable, which results in storage difficulties. There is need to find an economic way of dealing with cassava biotic stresses as this will help in the fight against hunger and poverty (Rudi, 2008).

2.9.1 Cassava mosaic disease

CMD is caused by a virus which belongs to the genus *Begomovirus* and family *Geminiviridae* (Busogoro et al., 2008). Most of the geminivirus genomes are bipartite; consisting of DNA-A and DNA-B (Abraham, 2012). DNA-A encodes functions associated with replication of the virus and encapsulation, while DNA-B is responsible for the movement functions (Harrison and Robinson, 1999; Abraham, 2012). CMD is caused by

several different geminiviruses including the ACMV, EACMV, EACMCV, EACMZV, EACMV (UgA), SACMV, *Madagascar cassava mosaic virus* (MCMV), *Indian cassava mosaic virus* (ICMV) and *Sri Lankan cassava mosaic virus* (SLCMV) (Malathi et al., 1987; Geddes, 1990; Hong et al., 1993; Zhou et al., 1997; Thresh et al., 1998). Apart from cassava, SACMV can infect *Arabidopsis* spp., *Phaseolus vulgaris* and *Malva parviflon* (Pierce, 2005; Abraham, 2012).

SACMV is closely related to EACMV type II Malawi isolates and EACMV Uganda isolates in DNA-A and DNA-B, respectively (Berrie et al., 2001). There is a possibility that recombination between the pre-existing viruses may result in new causal agents of the disease (Zhou et al., 1997) which may become more virulent. Among the neighbouring countries of Malawi, SACMV was first reported in Mozambique by Cossa (2011). EACMMV and EACMCV are also prevalent in Mozambique. Malawi is also known to have accommodated EACMMV, SACMV and EACMCV (Ogbe et al., 1997; Zhou et al., 1998; Alabi et al., 2011; Aloyce et al., 2013).

It is possible for a plant to be co-infected by different viral species (Busogoro et al., 2008), which means that it is possible to detect various virus species in one plant. It is also possible to have all -plants detected for mixed infections in a field (Busogoro et al., 2008). In this case plants tested for geminiviruses in a field might all be detected of mixed infection. CMD is known to occur in all cassava growing areas in Africa, Sri Lanka and India (Owor et al., 2004).

2.9.1.1 Losses due to cassava mosaic disease

According to Zhang et al. (2005), losses of cassava due to CMD in Africa have been estimated at 19.6-27.8% of the total production. The total crop yield losses were estimated at about US $ 1200-2400 million per annum (Thresh et al., 1997). CMD was reportedly the most wide spread of the virus diseases constraining production of cassava in sub-Saharan Africa (Ogbe et al., 2003).

2.9.1.2 Transmission of cassava mosaic geminiviruses

The whitefly, *Bemisia tabaci*, is a vector that transmits CMD causing viruses (Fargette and Vie, 1995); however, the use of cuttings from previously grown plants that are infected with the viruses contribute to the spreading of the disease (Busogoro et al., 2008). A study conducted by Mabasa (2007) found that a higher percentage (27.1%) of CMD infection

was due to the use of infected planting materials compared to whitefly borne-infections (10.4%). CMD is not seed transmissible (Storey and Nichols, 1938).

2.9.1.3 Symptoms of cassava mosaic disease

CMD symptom expression is influenced by a number of parameters such as host genotype, growing season, virus species causing the disease and stage of crop growth (Busogoro et al., 2008; Adjata et al., 2011). Plants with mixed infections of CMD begomoviruses are reported to have severe symptoms (Ogbe et al., 2006). As indicated in Figure 2.2 the symptoms of the disease are yellow mosaics, mottling, misshapen and twisted leaflets, overall reduction of leaf sizes and plant size, producing few or no tubers (Legg and Thresh, 2003; Alabi et al., 2011). CMD reduces photosynthetic area with consequent reduction of shoot and root development and growth as it may infect cassava plants as early as one month after planting (MAP), leading to reduction in size of leaves (El-Sharkawy, 1993). This leads to reduction in root yield and yield losses of up to 90% have been reported (Hahn et al., 1980).

Figure 2.2 Cassava leaves with cassava mosaic disease symptoms (Photo by I. Benesi, 2013)

2.9.2 Cassava brown streak disease

CBSD is caused by a single stranded RNA (ssRNA) virus of the genus *Ipomovirus* and family *Potyviridae* (Monger et al., 2001; Mbanzibwa et al., 2009). The *Potyviridae* family is comprised of the biggest number of positive ssRNA plant viruses (Mbanzibwa et al., 2009).

Economically, CBSD is one of the major damaging biotic constraints to cassava production in Africa (Bigirimana et al., 2011). Unlike CMD, CBSD expresses symptoms on stems and roots apart from leaves. It has been reported in Kenya, Tanzania, Mozambique, Zambia, Malawi and Uganda, but the incidence and effects are greatest in the lowland coasts of Kenya, Mozambique and Tanzania (Hillocks et al., 2002). It was first reported in Tanzania by Storey (1936), but in Malawi it was first reported by Nichols (1950). In Mozambique it was reported in 1999 (Zacarias and Labuschagne, 2010). It is caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak* virus (UCBSV) both of which have been reported in Malawi (Mbewe et al., 2014). UCBSV is widely distributed than CBSV. There is greater genetic diversity among UCBSV as compared to CBSV isolates.

2.9.2.1 Losses due to cassava brown streak disease

With CBSD infection, root necrosis is more important than shoot infection and losses due to CBSD are more due to loss of root quality than root weight and can cause losses of up to 100% (Nichols, 1950; Pariyo et al., 2015). In most susceptible cultivars, root necrosis is visible at five or more MAP during which the plant had undergone root development and tuberisation (Kulembeka, 2010). Root yield losses may reach 60-70% in susceptible cultivars (Zacarias and Labuschagne, 2010).

2.9.2.2 Transmission of cassava brown streak viruses

CBSD is transmitted by the whitefly (*B. tabaci*) just like CMD (Czosnek et al., 2001; Maruthi et al., 2005). However, the use of infected vegetative propagated materials play a big role in its spread. Apart from transmitting the virus, whiteflies feed on phloem sap and excrete honey dew that promotes growth of fungi (Brown and Czosnek, 2002). CBSD is not seed transmissible (Rwegasira and Chrissie, 2015).

2.9.2.3 Symptoms of cassava brown streak disease

Plants infected with CBSD may show symptoms in the leaves, stems and roots. The root symptoms of CBSD include the yellow-brown, corky necrosis in the parenchyma (starch bearing tissue), resulting in roots that are unfit for consumption as indicated in Figure 2.3. The roots have also yellow blotchy chrolosis. The symptoms of CBSD can sometimes be masked by symptoms of CMD, making it difficult to identify the disease in the field (Bock and Guthrie, 1976). Foliar symptoms of CBSD are yellow chlorosis, irregular blotchy chlorosis and roughly circular patches that are more prominent on old leaves than young ones (Mohammed et al., 2012; Hillocks and Thresh, 2000). As such, there is less reduction

or damage in shoot growth compared to CMD infection. CBSD can also be caused by mixed infection (Mbanzibwa et al., 2009).

Figure 2.3 Cassava leaves with cassava brown streak disease symptoms (Ntawuruhunga and Legg, 2007)

2.9.3 Control of cassava mosaic and brown streak diseases

There are a number of approaches that have been employed to overcome CMD and CBSD in Africa; of which some are phytosanitation and the introgression of host resistance to develop varieties that would withstand the two viral diseases (Thresh and Otim-Nape, 1994) through plant breeding. Disease resistance breeding is one of the approaches that is promising in the fight against these diseases.

Phytosanitation generally helps to decrease the availability of sources of infection from which clean plants can contract the disease through transmission by whiteflies or human activities such as the use of infected cuttings for propagation (Thresh et al., 1998). During phytosanitation, diseased plants are removed to prevent further spread of the virus. Phytosanitation includes crop hygiene, the use of virus free planting materials, and rogueing (Thresh et al., 1998). The use of disease-free planting materials has been known to be one of the strategies in the management of the disease (Hillocks et al., 2001; Hillocks and Jennings, 2010). In cultivars which manifest foliage symptoms in the case of CBSD, the collection of healthy stems may be supplemented with rogueing of symptomatic plants at sprouting (Hillocks and Jennings, 2010).

2.10 Cassava breeding

Cassava breeding involves the process of introduction, development and identification of new cassava genotypes (Were, 2011). All *Manihot* species are diploid with a chromosome number of 2n = 36 (De Carvalho and Guerra, 2002; Jennings and Iglesias, 2002). Introgression of genes from wild species has been beneficial to cassava breeding.

2.10.1 Natural and artificial hybridisation

Cassava can be crossed naturally or artificially. Natural crossing is done by insect pollinators. Due to cassava pollen being sticky, wind pollination is almost impossible. The sexually compatible wild relatives can be either the species that are closely related to cultivated cassava or its immediate ancestors (Chavarriaga-Aguirre and Halsey, 2005). Genetic and physiological factors seem to influence the gene flow from cassava to related populations (Chavarriaga-Aguirre and Halsey, 2005).

Cassava is selected based on the ability to pass on good traits to the progeny or recombination to give superior genotypes for the specific trait of interest (Ceballos et al., 2004). Varieties which are genetically diverse for preferred traits when crossed, produce F1 hybrids with high heterosis (Falconer and Mackay, 1996; Sleper and Poehlman, 2006).

2.10.2 Cassava polyploidy

Cassava ploidy levels, which refer to the number of copies of the entire chromosome set in a cell of an organism (Acquaah, 2007) plays a crucial role in cassava breeding. Though it is diploid, it can also be polyploid. Cassava polyploidy breeding has contributed to breeding for yield and stress tolerance (An et al., 2014). Polyploid crops that are influenced by the size of the nuclear genome are reported to have larger cells than diploid ones (Nassar et al., 2008), and this affects cell volumes and anatomical structures (An et al., 2014). De Carvalho and Guerra (2002) reported chromosome sizes of 1.23 to 2.41 μm per karyotype with an average length of 1.74 μm.

2.10.3 History of cassava breeding for disease resistance

Cassava breeding first began in Tanzania, formerly known as Tanganyika in 1935 at Amani Research Station during the early part of the 20th century (Jennings and Iglesias, 2002). Production of hybrids started in the 1930s when a large proportion of hybrids were produced through controlled pollination. The variations of the germplasm that existed then formed the basis for the growth of cassava production (Kawano, 2003). The landraces were improved for yield potential as well as pest and disease tolerance (Chikoti, 2011).

The overall objectives were to increase both yield per unit area and area under cultivation (Jennings and Iglesias, 2002).

True disease resistance in plants is genetic in nature and is usually manifested in two forms, namely inhibition of infection where a pathogen is prevented from infecting the plant and inhibition of growth of the pathogen where the pathogen infects the plant but its growth is suppressed; the first form is more common (Acquaah, 2007). In the past, breeding dealt very much with CMD rather than CBSD. Breeding for CMD resistance started during the early part of the 20th century at Amani Research Station in Tanzania (Legg and Fauquet, 2004).

In 1935, a British researcher by the name H. Storey, did extensive resistance breeding for CMD from rubber species x cassava hybrids (Nweke, 2009). However, the hybrids developed were poor yielding and had poor agronomic characteristics. Another researcher by the name Jennings, who headed the same research station from 1951, developed segregants from the cassava hybrid x rubber species which were observed to have higher resistance than the hybrids previously developed by H. Storey (Nweke, 2009). Cassava breeding is recognised as the appropriate long term solution in combating the disease (Legg and Fauquet, 2004) since efforts to develop control strategies such as phytosanitary measures, cultural practices, planting date, use of cultivar mixtures and insecticides have had limited success (Chikoti, 2011).

Breeding for disease resistance has been successful due to the relative ease of crossing cassava with closely related species such as *M. glaziovii*. The first resistance to CMD was recognised in backcross derivatives of *M. glaziovii* (Nweke, 2009). Though several studies have been done on breeding for CMD resistance in Africa and elsewhere, research on this is limited and the viruses keep on mutating; resulting in potent variants (Chikoti, 2011).

2.10.4 Mechanisms of disease resistance in cassava

Plants, including cassava, exhibit various defence mechanisms for protection against diseases such as CMD and CBSD. Six categories of resistance to CMD have been suggested and these are immunity, resistance to infection, resistance to virus establishment and spread within the host, resistance to multiplication of the virus, tolerance, and resistance to vectors (Hahn et al., 1980). The above mentioned mechanisms are interrelated (Chikoti, 2011). The cell to cell movement of ACMV into cassava plants parts of resistant and moderately resistant genotypes is restricted (Ogbe

et al., 2002). The ability of a plant to restrict virus movement and multiplication in resistant cultivars, results in appearance of inconspicuous or no disease symptoms (Chikoti, 2011).

Resistance to the insect vector is another resistance mechanism (Ogbe et al., 2002). This resistance to insect vectors is called avoidance, which can be explained as a mechanism by which the contact between the insect vector and the plant host is reduced (Acquaah, 2007). Although defence mechanisms have evolved over time, viruses also developed ways to overcome host plant defences (Chikoti, 2011). This can be due to recombinations which result in new viral strains (Zhou et al., 1997). The disease is best kept under control by the deployment of resistant varieties (Thresh et al., 1997).

Resistance to CMD was previously thought only to be polygenically or quantitatively inherited (Chikoti, 2011). Polygenic resistance is controlled by several genes with effects too small to be individually distinguished. Hahn et al. (1980) indicated the possibility of several genes being responsible for resistance to CMD. In addition to the landraces, wild species of cassava, including *M. glaziovii,* have been used since the 1930s for resistance breeding to CMD (Chikoti, 2011). However, resistance in landraces vary from moderately resistant to resistant (Jennings and Iglesias, 2002). Varieties like Namikonga in Uganda and Kaleso in Tanzania have been identified as resistant to CBSD based on both virus quantities and disease severity symptoms (Kiweesi et al., 2014; Maruthi et al., 2014). In response to virus invasion, some plants express antiviral inhibitors that block the transmission and interfere with replication and translation of viruses (Bellows and Fisher, 1999). This leads to reduction in virus quantities in a plant.

Molecular techniques like Bulked Segregant Analysis (BSA), Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphism (SNP) have been used to develop molecular markers associated with resistance genes. NS169, NS158, SSRY28, SSRY040 and RME1 are some of the markers used in CMD resistance studies (Akano et al., 2002; Carmo et al., 2015). Two genes are associated with cassava resistance and these are CMD1 and CMD2. CMD2 is a dominant monogenic resistance gene and has been discovered in a number of African landraces (Akano et al., 2002; Rabbi et al., 2014). The first host-plant resistance was found in the third back cross derivative of an interspecific cross between *M. esculenta* and *M. glaziovii* (Akano et al., 2002). Transgenic plants have shown high resistance to CBSD with respect to viral load and symptom severity. CBSV-CP hairpin construct generates immunity against both CBSV and UCBSV (Vanderschuren et al., 2012).

Viral disease resistance of a crop variety is measured using a number of methods. The methods include disease severity scoring, disease incidence calculation, viral load quantification, yield reduction and crop growth and development assessment (Sseruwagi et al., 2004; Rwegasira and Rey, 2012; Mohammed et al., 2012; Kiweesi et al., 2014; Maruthi et al., 2014). A number of studies have attributed low viral load and low disease severity to resistant cassava varieties (Kiweesi et al., 2012; Maruthi et al., 2014). This means that resistant varieties show less disease symptom severity and low virus quantities.

2.11 Nucleic acid diagnostics

Nucleic acid extraction from infected plant cells is the first step in virus detection. There are a number of methods used to extract nucleic acids (Nishiguchi et al., 2002). Cetyltrimethyl ammonium bromide (CTAB) and Sodium dodecyl sulphate (SDS) extraction methods have been reported to be relatively efficient in terms of the amount of nucleic acids produced, apart from being simple to use and having good intensity of fragments when analysed by PCR (Méndez et al., 2010). These methods have been used successfully for crops like cassava and tomato. The CTAB method may be used with either fresh or dehydrated plant material. Secondary metabolites such as polysaccharides pose a challenge in nucleic acids extraction (Sarwart et al., 2006). DNA and RNA can be extracted either separately or simultaneously as total nucleic acids in both fresh and dry samples (Rubio-Piña and Zapata-Pérez, 2011; Abarshi et al. 2012; Devi et al., 2013; Sun et al., 2014). However, the method used for extraction needs to take into consideration the polyphenol and polysaccharide content in a plant (Rubio-Piña and Zapata-Pérez, 2011).

2.11.1 Polymerase chain reaction

PCR is a DNA based technology which was invented in 1984 (Mullis and Faloona, 1987). PCR revolutionised basic research in molecular diagnosis and biotechnology in terms of detection of a variety of pathogens (Abarshi, 2012). This is an *in vitro* method used to detect DNA present in a sample by exponential increase of DNA fragments with known end sequences (Saiki et al., 1985). This technique ensures that there are many copies of specific DNA fragments from initially very small quantities (Erlich, 1989). PCR is versatile, sensitive and specific (Aloyce et al., 2013).

PCR technology involves three major steps which are denaturation, annealing and extension (Erlich, 1989). Denaturation involves subjecting the template DNA to high temperatures, usually 90-97°C so that the strands can separate to single strands (Erlich,

1989; Joshi and Deshpande, 2011). During the second step (annealing), primers anneal to the complementary single DNA strands at a lower temperature of 50-60°C. Extension is the final step. During extension, an enzyme, Taq DNA polymerase, synthesizes a complementary nucleotide strand following a particular sequence using the nucleotides available from dNTPs. For most PCRs, extension occurs at 72°C. During PCR a thermal stable enzyme DNA polymerase is used (Erlich, 1989; Joshi and Deshpande, 2011). This process is done repeatedly.

2.11.2 Real-time PCR

Real-time qPCR is a modification of PCR. This molecular technique is used to detect and quantify DNA in a given sample. The technique has helped in the detection and quantification of both CBSV and CMGs (Moreno et al., 2011; Rajabu, 2013). It is quick, easy to use, has lower chances of contamination, has high sensitivity and allows one to monitor the progress of the reaction in real time (Fox, 1990; Kokkinos and Clark, 2006; Schmittgen and Livak, 2008; Jensen, 2012). However, real time PCR is costly and requires skilled labour which poses a challenge to many African laboratories (Fauquet et al., 2003; Ogbe et al., 2003).

Real-time qPCR is abbreviated in a number of ways that include the following: RTQ-PCR, Q-PCR, qRT-PCR, or qPCR. Some confuse RT-PCR with real-time PCR; but this stands for reverse transcription PCR used when the starting template is RNA (Jensen, 2012). When real-time PCR is used with the initial template of RNA, as is the case with CBSV, the terms given are reverse transcription quantitative PCR (RT-qPCR) and real-time reverse transcription PCR (real-time RT-PCR) (Qiagen, 2003; Jensen, 2012). RT-qPCR can be performed in one or two steps depending on whether all the reagents have been added in a single tube where reverse transcription (RT) and quantification is done or RT and quantification are done separately (Qiagen, 2003). RT is also called cDNA synthesis. When the starting material is DNA, the term used is either qPCR or other terms mentioned above without including RT since there is no cDNA synthesis involved. Since Begomoviruses are DNA viruses (Harrison and Robinson, 1999), they do not need to undergo reverse transcription, while CBSV are RNA viruses (Monger et al., 2001) and need to undergo reverse transcription before quantification can be done either in one or two steps.

Real-time qPCR has become a widely used technique for quantifying gene expression (Bolha et al., 2012). There are two main methods used for data analysis from real-time qPCR experiments; these methods are absolute and relative quantification methods (Livak

and Schmittgen, 2001). In absolute quantification, the input copy is determined by relating the PCR signal to a constructed standard curve, while in relative quantification PCR signal of the target is related to the PCR signal of another sample. The $\Delta\Delta$CT method (Delta-delta Ct method) is a method commonly used for analysis of changes in gene expression (Livak and Schmittgen, 2001) and uses arithmetic formula to achieve the results of relative quantification (Bolha et al., 2012).

Relative virus quantity are calculated as follows:

$$\text{Relative quantity} = 2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$$

$$\Delta CT_{sample} = CT_{\text{gene of interest in sample}} - CT_{\text{reference gene in sample}}$$

$$\Delta CT_{calibrator} = CT_{\text{gene of interest in calibrator}} - CT_{\text{reference gene in calibrator}}$$

RQ is the relative virus quantity and CT is the threshold cycle (Livak and Schmittgen, 2001; Čikos et al., 2007). There is no need for the use of gel-electrophoresis in qPCR as both detection and quantification are done in a single tube (Kubista and Zoric, 2004; Bustin and Mueller, 2005).

The application of qPCR, depends on the protocol used. Generally, two chemistry protocols are used and these are TaqMan and SYBR Green I (Roche Applied Science, 2006; Hernández-Rodriguez and Ramirez, 2012). The reagents used in these protocols are distinct. The major difference between the two is that the TaqMan assay requires the use of sequence specific labelled probes which gives it an advantage over SYBR Green I as it is very specific primer dimers. Any other non-specific amplifications are not detected in TaqMan assay while SYBR Green I does not use probes, as it binds to any double stranded DNA (Roche Applied Science, 2006).

When the TaqMan assay is used, the DNA polymerase enzyme cleaves the target specific hybridisation probe and releases the reporter dye (TET or FAM) from the probe (Gibson et al., 1996); and this cleavage separates the reporter and quencher dyes (Applied Biosystems, 2010). When quantities of DNA increase, the emission of the reporter fluorescence also increases (Gibson et al., 1996); but the increase occurs only when the target sequence is complementary to the probe (Applied Biosystems, 2010). Fluorescence emission is monitored, in real time, using a sequence detector (Gibson et al., 1996).

Reference genes are used to normalise gene expression (Livak and Schmittgen, 2001). The reference gene represents DNA quantity and quality in each sample (Bustin and Mueller, 2005; Moreno et al., 2011). If there is a high quantity of the copies of the target at the beginning of the reaction, the number of amplification cycles needed for detection is

reduced (Bustin and Mueller, 2005). Cytochrome oxidase (COX), ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) and L2 ribosomal protein are the common reference genes that have been used in qPCR of CBSVs and CMGs (Nassuth et al., 2000; Alabi et al., 2008; Abarshi, 2012; Mohammed, 2012; Adams et al., 2013; Ogwok et al., 2015).

The internal control for gene normalisation can be designed in several ways, including scrambling of the internal sequence, mutation of the target amplicon, deletion/insertion of sequences into the targeted amplicon or splicing of the sequences of the target primer on a non-homologous DNA sequence (Gibson et al., 1996). The target and control should use the same primers, should contain equivalent amounts of guanine and cytosine nucleotides, and should have the same length. Absolute quantitation requires the accurate determination of internal control concentration, while relative quantitation can be established easily with a validated internal control (Gibson et al., 1996).

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CHAPTER 3

Quantification of cassava brown streak viruses and mosaic geminiviruses in F1 cassava progenies in the field

3.1 Introduction

CBSD is caused by CBSV and UCBSV which are single stranded RNA (ssRNA) viruses in the family *Potyviridae*; genus *Ipomovirus*. CBSD symptoms include leaf discoloration, and chlorosis and brown streaks on the leaf and stem. Most importantly, a yellow to brown dry necrotic rot is produced in the roots of severely affected plants which renders them unusable. The disease is primarily spread through infected planting materials, but Maruthi et al. (2005) and Ntawuruhunga and Legg (2007) attributed the spread to the whitefly vector, *Bemisia tabaci* Genn. CBSD alone has been reported to cause up to 70% yield loss in Tanzania (Hillocks et al., 2001) and 40% in Malawi (Gondwe et al., 2003).

CMD is caused by cassava mosaic geminiviruses belonging to the genus *Begomovirus* and family *Geminiviridae* (Busogoro et al., 2008). Losses due to CMD in Africa were estimated at 19.6-27.8% of the total production (Zhang et al., 2005). The virus causing CMD is transmitted by whitefly, *B. tabaci* (Fargette and Vie, 1995), however, the use of cuttings from infected plants is also a common way of spreading the disease (Busogoro et al., 2008). A study conducted by Mabasa (2007) found higher disease incidence due to the use of infected planting materials compared to whitefly borne-infections. Symptoms of the disease include yellow or pale green chlorotic mosaic of leaves, commonly followed by distortion or crumpling. In severe cases the plants become stunted and the petioles immediately below the shoot tip may be angled downwards, and occasionally may become necrotic, shrivel and abscise (Legg and Thresh, 2003). The chlorosis of the leaves is likely to affect photosynthetic activities in a plant. In Africa, the disease is caused by the ACMV, EACMV, EACMCV, EACMZV, EACMV (UgA), SACMV and MCMV) (Geddes, 1990; Hong et al., 1993; Zhou et al., 1997; Thresh et al., 1998).. In Malawi, EACMMV, EACMCV, SACMV, CBSV and UCBSV are known to occur (Ogbe et al., 1997; Zhou et al., 1998; Mbanzibwa et al., 2009; Alabi et al., 2011; Aloyce et al., 2013; Mbewe et al., 2014).

The destructive effects of CMD and CBSD have lead the science community to put more effort into the fight against them, instead of concentrating on yield improvement (Hyman et al., 2012) which is a draw back to the fight against hunger. A number of approaches have been employed in the control of CMD and CBSD, of which one is breeding of resistant varieties (Thresh and Otim-Nape, 1994; Shaba et al., 2002). Genetic improvement is a

good approach that may confer a long term solution to the diseases (Hyman et al., 2012). It is essential to identify cassava genotypes that tolerate these two diseases, either by controlling the multiplication of viruses causing the two diseases, or preventing the symptoms. Genotypes that suppress both virus replication and symptom expression would be good candidates for disease resistance studies. It would be crucial to combine disease resistance with farmer preference in breeding, as some farmers prefer sweet cassava while in some places they prefer bitter varieties, depending on the intended use (Benesi, 2005). Knowing the response of cassava plants to CMD and CBSD, symptom expression and virus concentration is a good approach to selecting genotypes to advance in the breeding programme; this would lead to identification of the right materials for further evaluation. It is also of importance to know which virus species infect the plants, causing the most symptoms and high viral concentrations, and which genotypes are likely to be infected by a particular virus species.

Due to the magnitude of the destruction CMD and CBSD are causing, there are a number of projects that have been undertaken in order to mitigate the effects of the two diseases. Recent projects such as the "New cassava varieties and clean seeds to combat CMD and CBSD" and "Cassava diagnostics" are being conducted in Malawi in order to develop resistant/tolerant varieties and find other means of overcoming these challenges. The aims of this study were therefore to identify F1 crosses that are resistant/tolerant to CMD and CBSD, and to correlate the disease severity symptoms and the virus quantities. Disease resistance is the ability of a plant to withstand pathogen attack. In some cases the plant may be attacked by the pathogen but the pathogen multiplication and movement is restricted. This leads to low pathogen quantity levels (Hull, 2009). Some studies have reported high CBSV and UCBSV levels in plants but low disease symptom expression while others have exhibited both low virus levels and low disease symptom expression. Plants with low virus quantities and low symptom severity expression are regarded as resistant/tolerant (Kiweesi et al., 2014; Maruthi et al., 2014).

3.2 Materials and methods

3.2.1 Experimental sites

The field experiment was conducted at Chitala Research Station from November, 2013 to December, 2014. Chitala Research Station is found in the lake shore district of Salima. It is located at 13° 40' S and 34° 15' E. It has an altitude of 513 m above sea level. The topsoil is dark to very dark reddish brown and varies considerably in texture from place to place, between sandy loam and sandy clay (Brown and Young, 1965). Natural field

inoculation was used. There is prevalence of CMD and CBSD by natural infestation in this site (Ogbe et al., 1997; Zhou et al., 1998; Mbanzibwa et al., 2011; Mbewe et al., 2014). In addition, Chitala is a high pressure area for both CMD and CBSD and because of this most of the cassava disease screening trials are conducted there.

3.2.2 Parental genotypes

A number of parents in the crossing block which is situated at Chitedze Research Station were used to produce seeds from crossing. The cassava crossing block contained the following genotypes: Chamandanda, Phoso, Mulola, 01/1569, Beatrice, 01/1316, Silira, Mbundumali, Kachamba, MK06/0323, Chiombola, Maunjili, Sauti, Sagonja, Yizaso and Gushe (Table 3.1).

The parental genotypes were selected based on the levels of resistance/tolerance or susceptibility to CMD, CBSD and other diseases and pests. Furthermore, the choice of parents was also made based on the yield and taste as these are also factors that influence utilisation and cultivation by farmers. However, the use of the F1 progenies for this field trial depended on the amount of seeds collected, the number of seedlings successfully raised in the glasshouse and the ability of the seedlings to survive in the field.

In this experiment F1 cassava progenies derived from the locally available genotypes were tested so as to identify which progenies perform better than others in terms of CMD and CBSD symptom severity as well as virus quantities. The use of locally available genotypes ensured that the parents have the other desired traits required by the local communities. Simultaneous collection of both molecular, symptom severity and incidence data will give a better basis of the kind of materials that are needed for further evaluation.

3.2.3 Cassava crossing and seed collection

Seeds from the open pollinated (OP) plants were obtained from the crossing block at Chitedze Research Station. The plants in the crossing block were left to pollinate naturally and they were monitored for maturity. Physiologically mature fruits were collected in sampling bags before shattering. Each bag was labelled with the name of the F1 progeny produced. The open pollinated seeds were obtained from the following seven maternal parents: Mbundumali, Chamandanda, Silira, Kachamba, Beatrice, MK06/0323 and Gushe; therefore the F1 progenies produced were labelled with the maternal name and an "OP" at the end. However, pollen might have come from any of the genotypes in the crossing block in what might be referred to as a polycross. After collection, the fruits were taken to

Table 3.1 Tolerance/susceptibility to cassava mosaic and cassava brown streak diseases of the parental genotypes used in crosses

Variety	Category	Release	Yield (ton ha^{-1})	Taste	Disease resistance/tolerance
Mbundumali	Local selection	1980s	25	Sweet	Tolerant to CBSD and CGM but susceptible to CMD
Silira	Improved	1999	25	Bitter	Tolerant to CMD and CM, susceptible to CGM
Phoso	Improved	2008	35	Bitter	Tolerant to CMD and CBSD
Mulola	Improved	2008	40	Bitter	Tolerant to CMD, CM and CGM
Kalawe	Improved	2008	Not published	Sweet	Moderately resistant to CMD and CBSD
Kachamba	Improved	-	Not published	Sweet/ bitter	Susceptible to CMD but resistant/moderately resistant to CBSD
Chamandanda	Improved	2010	Not published	Sweet	Tolerant to CMD
Beatrice	Improved	Clone	Not published	Bitter	Tolerant to CMD
01/1316	Improved	Clone	Not published	Bitter	Promising - under evaluation
01/1569	Improved	Clone	Not published	Bitter	Promising - under evaluation
MK06/0323	Improved	Clone	Not published	Bitter	Promising - under evaluation
Yizaso	Improved	2002	25	Bitter	Tolerant to CMD, CGM and CM
Chiombola	Improved	2009	45	Bitter	Tolerant to CM and CGM
Sauti	Improved	2002	25	Bitter	Tolerant to CMD, CGM and CM
Sagonja	Improved	2009	25-35	Bitter	Tolerant to CMD, CBSD, CGM and CM
Maunjili	Improved	1999	35	Bitter	Tolerant to CMD, CGM and CM
Gushe	Local	-	-	Bitter	Tolerant to CMD and CBSD

CBSD – Cassava Brown Streak Disease; CGM – Cassava Green Mite; CM – Cassava Mealy Bug; CMD – Cassava Mosaic Disease

Haggblade and Zulu (2003); Mkumbira et al. (2003); USAID (2011); Alene et al. (2013)

the laboratory for processing. The collected F1 seeds were planted in floating trays in a glasshouse. After one month they were transplanted to the field at Chitala Research Station.

There were five seedlings per cross progeny which were replicated three times in a randomised complete block design (RCBD). The seedlings were planted on ridges with a spacing of 90 cm x 50 cm. Leaf samples from Mbundumali and Mulola parental genotypes were tested in the Biotechnology laboratory at Chitedze Research Station for the presence of viruses causing CMD and CBSD using PCR and gel electrophoresis. Cuttings from plants that tested negative were planted along with the F1 progenies as controls. Mbundumali was used as susceptible control to CMD but a tolerant control to CBSD. Mulola was used as a susceptible control to CBSD but a tolerant control to CMD.

The recommended agronomic practices in Malawi were applied. The seedlings were left to grow in the field for nine months, and then CMD and CBSD severity and incidence scoring was done in the field. Leaf samples were collected for laboratory analysis to determine the presence and quantities of CMGs and CBSVs. Thereafter, scoring and leaf collection for laboratory analysis were done concurrently every month for three subsequent months making it a total of four months of sampling and disease scoring. Monthly rainfall data was collected during the entire research period as shown in Table 3.2.

3.2.4 Leaf sampling, disease incidence and severity

Foliar disease severity was observed visually in each of the plants and severity scores were based on a scale of 1-5; where 1 was scored for a symptomless plant and 5 for plants with the most severe symptoms (Hahn et al., 1980; Sseruwagi et al., 2004; Rwegasira and Rey, 2012) as shown in Table 3.3. CBSD symptoms on the stem appear as brown streaks on the greener upper tip portions of the stem and when present were recorded as a "+" or "-" for asymptomatic stems. Data was collected starting from nine MAP up to twelve MAP. Disease incidence was calculated from five plants per cross replicate as follows:

Incidence %= Number of plants with disease symptoms/Total number of plants x 100 (Sseruwagi et al., 2004).

Table 3.2 Rainfall data from November 2013 to December 2014 for Chitala and Chitedze Research Stations

Site	Month														Total
	Nov 2013	Dec 2013	Jan 2014	Feb 2014	Mar 2014	Apr 2014	May 2014	Jun 2014	Jul 2014	Aug 2014	Sep 2014	Oct 2014	Nov 2014	Dec 2014	
Chitedze	27.1	112.1	198.4	290.2	24.2	65.1	14.1	0.0	0.0	0.0	0.0	5.9	17.8	61.0	815.9
Chitala	14.2	107.9	177.1	344.2	121.6	4.9	0.0	0.0	0.0	0.0	0.0	10.61	1.4	143.67	925.58

Source: Chitedze Meteorological Station; Chitala Research Station

Table 3.3 Foliar symptom severity scale for cassava mosaic and cassava brown streak diseases

Scale	Description	
	CMD	**CBSD**
1	No symptoms	No symptoms
2	Mild chlorosis, mild distortions at the bases of most leaves, while the remaining parts of the leaves or leaflets appear green and healthy	Slight chlorotic spots on leaves or stems
3	Mosaic patterns on most leaves, narrowing and distortion of lower one-third of the leaflets	Chlorotic spots easily observable on leaves or stems. Small lesions are observed on stems
4	Severe mosaic distortion of the thirds of most leaves and general reduction of leaf size and stunting of shoots	Severe chlorotic spots on leaves and severe necrotic lesions enlarged into streaks on stems
5	Very severe mosaic symptoms on all the leaves, distortion, twisting, misshapen and severe leaf reduction of most leaves accompanied by severe stunting of plants	Very severe chlorotic/ necrotic blotches and leaf wilt. Severe necrotic lesions, streaks, withering and die-back on stems

Sseruwagi et al. (2004); Rwegasira and Rey (2012)

Leaf samples for laboratory analysis were collected by taking the third fully expanded leaf of the sampled plant (Abarshi et al. 2012; Kumar et al., 2014). The leaves were collected from five plants per cross. After collection, the leaves were preserved in a herbarium plant press and taken to the laboratory for analysis. All the plants which were used for disease severity scoring in each treatment were also used for disease incidence calculation and nucleic acid extraction for virus quantification.

3.2.5 Nucleic acids extraction and bulking

3.2.5.1 RNA extraction

Total nucleic acid (TNA) was extracted from leaf samples using the CTAB (hexadecyltrimethylammonium bromide) protocol modified by Maruthi et al. (2002) and described by Abarshi et al. (2012). CTAB extraction buffer with a volume of 750 µl [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA (ethylene-diaminetetraacetate), 100 mM Tris-HCl (pH 8.0)] was added to approximately 100 mg of dried cassava leaf sample and was ground using a mortar and pestle. The ground mixture was transferred to a 1.5 ml microcentrifuge tube. The contents were vortexed briefly and incubated at 60°C in a water bath for 30 min and mixed by inverting. A volume of 750 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added and mixed thoroughly by vortexing. Samples were centrifuged at 13000 g for 10 min. Nucleic acids from the aqueous phase was precipitated in a 300 µl cold isopropanol and the contents were mixed gently and incubated at -20°C for 60 min. This was followed by centrifuging the contents at 13000 g for 10 min at 4°C. The supernatant was discarded without disturbing the DNA pellet. The pellets were washed in 500 µl with 70% ethanol by centrifuging at 13000 g for 5 min.   The ethanol was carefully discarded. The tubes were allowed to air dry at 23°C for at least 30 min. Finally, the nucleic acid pellets were resuspended in 100 µl sterile distilled water.

Reaction buffer (10 X) [100 mM Tris-HCl (pH 7.5 at 25°C), 25 mM $MgCl_2$, 1 mM $CaCl_2$)] was added to the tube with DNase I and nuclease free water. The mixture was incubated for 30 min at 37°C. One µl of 50 mM EDTA was added and incubated again at 65°C for 10 min. The extracted RNA from the five plants per replicate of each cross were bulked. During bulking RNA was pooled into a new microcentrifuge tube by taking 30 µl of RNA from each plant sample in a replicate. This was done in order to have a single composite sample for each replicate.

3.2.5.2 DNA extraction

DNA was extracted from dried leaf samples using a modified CTAB protocol as described in section 3.2.5.1. After extraction, the nucleic acid was resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 1 µl of 10 mg ml^{-1} of RNase A. DNA Bulking was done as described in section 3.2.5.1.

3.2.6 Complementary DNA synthesis

Since CBSV and UCBSV are RNA viruses, cDNA was synthesized before quantification. Template RNA (2 µl) and 0.5 µl Oligo (dT) primers were pipetted into a 1.5 ml Eppendorf tube containing 10 µl of nuclease-free water to a total reaction volume of 12.5 µl. The contents were gently mixed, centrifuged briefly and incubated at 65°C for 5 min. The mixture was centrifuged and placed on ice. The following were added to the tube: 4 µl of 5 x reaction buffer, 0.5 µl of Thermo Scientific^{TM} Ribolock^{TM} RNase inhibitor (20 U), 2 µl of 1 mM of dNTP mix and 1 µl of RevertAid H Minus reverse transcriptase (200 U) (Thermo Scientific) to make a final volume of 20 µl. The components were gently mixed and centrifuged. The mixture was incubated at 55°C for 60 min. Then it was heated for 10 min at 70°C. The synthesized cDNA was stored at -20°C awaiting quantification.

3.2.7 Quantification of *cassava mosaic geminiviruses* and *cassava brown streak viruses*

3.2.7.1 Quantification of *Cassava brown streak virus* and *Ugandan cassava brown streak virus*

Quantification of the CBSV and UCBSV titre in cassava progenies was done using TaqMan with probes and their complementary primers that amplify fragments ranging from 75 bp to 200 base pairs (bp) of the coat protein (Adams et al., 2013; Ogwok et al., 2015). A reference gene, Cytochrome oxidase (COX) was used as internal control for data normalisation (Adams et al., 2013; Kaweesi et al., 2014). ROX [25 µM solution of 5-carboxy-X-rhodamine in 10 mM Tris-HCl (pH 8.6), 0.1 mM EDTA, and 0.01% Tween$^{®}$-20] was used as a passive reference. qPCR reactions contained 12.5 µl of Maxima probe qPCR mastermix (2X) (Thermo Scientific), 1 µl of 7.5 µM forward and reverse primers, 0.5 µl of 5 µM probe, 0.5 µl of 15 µM Rox, 1 µl of cDNA template and 9.5 µl of sterile distilled water in a final volume of 25 µl. Sterile distilled water was used as a no template control, and there were two positive controls from samples that previously showed amplifications of CBSV and UCBSV. The mixture was dispensed into a 48-well PCR plate and run on a Step One^{TM} Real –Time PCR system (Applied Biosystems). The qPCR plates were sealed using adhesive PCR seals to provide protection against evaporation and dispersion. The

$\Delta\Delta$Ct method was used for quantification. Ct values obtained from the real time PCR were used to calculate relative virus titre. ΔCt was calculated as Ct_{target}-$Ct_{reference\ gene}$; $\Delta\Delta$Ct was calculated as $(Ct_{target}$-$Ct_{reference\ gene})_{calibrator} - (Ct_{target}$- $Ct_{reference\ gene})_{sample}$; therefore, relative virus titre was calculated as RQ= $2^{-\Delta\Delta Ct}$. The qPCR amplification programme was incubated at 48°C for 30 min, denatured at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and annealing/extension for 1 min at 60°C. Data was collected at 60°C stage. The primers, reference genes and probes used are shown in Table 3.4. Two technical replicates were used for each reaction. The experiment had three biological replicates. Due to the limitation of the number of wells, the biological replicates were performed on separate qPCR plates and an inter-run calibrator sample was used.

3.2.7.2 Quantification of *South African cassava mosaic virus* and *East African cassava mosaic Malawi virus*

Quantification of the SACMV and EACMMV was done using the SYBR Green I PCR mastermix (Thermo Scientific). Reference genes, ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and cytoplasmic ribosomal protein L2 were used as internal controls. RubisCOF and RubisCOR are 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 are designed to amplify a product size of 135 bp (Mohammed, 2012). Primers that amplify a 90 bp and 117 bp of AC1 gene of SACMV and EACMMV respectively were used for qPCR of the viruses (Table 3.5). The already designed primer sequences were obtained from the real-time qPCR tool of the Integrated DNA Technologies website (www.idtdna.com) using the following virus GenBank accessions: AJ006460 and AF155806. The primers were validated for EACMMV and SACMV specific amplification using DNA samples that were confirmed by PCR and gel-electrophoresis. qPCR reactions contained 8.5 µl sterile distilled water, 1.5 µl each reverse and forward primers (0.8 µM) and 12.5 µl maxima® SYBR Green/Rox (2X) and 1 µl DNA template in a final volume of 25 µl. Sterile distilled water was used as a no template control, and there were two positive controls from samples that previously showed amplifications of SACMV and EACMMV. A DNA sample of Beatrice OP at nine MAP was used as a calibrator sample.

Table 3.4 Primers and probes for *Cassava brown streak virus, Ugandan cassava brown streak virus* and the reference gene, cytochrome oxidase

Primer	Sequence (5'----->3')	Reference
CBSV-CpF	GCCAACTARAACTCGAAGTCCATT	Adams et al. (2013)
CBSV-CpR	TTCAGTTTAAGCAGTTCGTTCA	
UCBSV-CpF	GATYAARAAGACITTCAAGCCTCCAAA	Adams et al. (2013)
UCBSV-CpR	AATTACATCAGGRGTTAGRTTRTCCCTT	
COX-F	CGTCGCATTCCAGATTATCCA	Tomlinson et al. (2005)
COX-R	CAACTACGGATATATAAGRRCCRRAACTG	
CBSV-Cp-probe-FAM	/56-FAM/AGTCAAGGAGGCTTCGTGCYCCTC/3IABkFQ/	Adams et al. (2013)
UCBSV-Cp-probe-JOE	/56-JOEN/TCAGCTTACATTTGGATTCCACGCTCTCA/3IABkFQ	Adams et al. (2013)
COX-Probe-JOE	/56-JOEN/AGGGCATTCCATCCAGCGTAAGCA/3IABkFQ	Tomlinson et al. (2005)

The mixture was dispensed into Step One^{TM} Real-Time PCR system (Applied Biosystems) with a reaction plate of 48 wells. The qPCR plates were sealed using adhesive PCR seals to provide protection against evaporation and dispersion. The qPCR amplification programme was activated at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 10 sec. Data was collected at 72°C stage. The ΔΔCt (Delta-delta Ct) method was used for quantification. Ct values obtained from the real time PCR were used to calculate relative virus titre. ΔCt was calculated as Ct_{target}-$Ct_{reference\ gene}$; ΔΔCt was calculated as $(Ct_{target}$-$Ct_{reference\ gene})_{calibrator}$ − $(Ct_{target}$- $Ct_{reference\ gene})_{sample}$; therefore, relative virus titre was calculated as RQ= $2^{-\Delta\Delta Ct}$. Two technical replicates were used for each reaction. The experiment had three biological replicates. Due to the limitation of the number of wells, the biological replicates were performed on separate qPCR plates and an inter-run calibrator sample was used.

3.2.8 Data analysis

The CMD severity scores, incidence and relative virus titre were subjected to ANOVA (Analyses of variance) using GenStat 17.1 (Payne et al., 2014). Means were separated using least significant differences (LSD). Spearman rank correlation matrix was used to determine the correlation between CMD severity, incidence and EACMMV and SACMV titres.

3.3 Results and discussion

3.3.1 Cassava mosaic disease severity and incidence in F1 progenies from open pollinated crosses

After transplanting, seedlings were monitored for disease development relative to CMD trials in the preceding growing seasons. There was late CMD symptom development in the field trial. Therefore sampling was delayed until nine MAP in order to reduce chances of disease escape by plants and to observe and elucidate the relationship between CMD symptom severity, incidence and virus titre which could not be achieved if data on one of these was unavailable. There is a possibility that the late development of disease symptoms might have been due to unfavourable environmental conditions toward whitefly vector activities and multiplication of viruses. This might be the case because CMD development is affected by environmental conditions and may vary depending on location of the field and year of cultivation (Colvin et al., 2004; Adjata et al., 2012; Sing'ombe et al., 2015).

Table 3.5 Primers for *East African cassava mosaic Malawi virus*, *South African cassava mosaic virus*, ribulose-1,5-bisphosphate carboxylase oxygenase and L2 ribosomal protein

Primer	Sequence (5'----->3')	Reference
EACMMV F	TCGTCTTCCCTGTCCTACTATC	www.idtdna.com
EACMMV R	CACACGTCCCAGACGAAATAG	
SACMV F	GCTGTCGGCCAGTCATATT	www.idtdna.com
SACMV R	GTTGACACGCTCCACTACTT	
RubisCol F	CTTTCCAAGGCCCGCCTCA	Nassuth et al. (2000); Alabi et al. (2008); Alabi (2009); Abarshi (2012)
RubisCol R	CATCATCTTTGGTAAAATCAAGTCCA	
L2F	TGGTGTTGCCATGAACCCTGTAGA	Mohammed (2012)
L2R	CGACCAGTCCTCCTTGCAGC	

Fargette et al. (1993) attributed low CMD development to low growth rate of the cassava plants. To some extent this might have been the case in this experiment because plant growth rate observed was not as expected when compared to plants in the surrounding trials where cuttings were used instead of botanical seeds and by the time of final disease scoring and sampling at 12 MAP, the plants were still growing. However, there is no data on crop growth because this research was not intended to investigate the growth habits including morphological and physiological characteristics of the F1 progeny crosses. Apart from the above mentioned reasons, the late symptom development might have been due to low disease pressure during the growing season in the location since the susceptible control was not different.

Disease scoring and leaf sampling were done simultaneously each month from nine to twelve MAP. The ANOVA results for CMD severity of F1 progenies from OP crosses (Table 3.6) showed that at p=0.05 Mbundumali OP, Kachamba OP crosses and a susceptible control (Mbundumali) were similar at nine MAP. However, Mbundumali and Kachamba OP crosses were significantly different from a tolerant control (Mulola) and the rest of the F1 progeny crosses. Mbundumali OP and Kachamba OP crosses had the highest CMD severity score (1.47). There were no disease symptoms observed from Gushe, Chamandanda, MK06/0323 and Silira OP crosses.

At ten MAP, Mbundumali OP and Kachamba OP crosses were similar with severity scores of 1.8 respectively; but were significantly different from both tolerant and susceptible controls and the rest of the progeny crosses. There were no disease symptoms observed from Gushe, Chamandanda, MK06/0323 and Silira OP crosses.

At eleven MAP, Mbundumali OP cross and Mbundumali (control) were similar with severity scores of 2.20 and 2.00 respectively; but they were significantly different from the tolerant control and the rest of the F1 progeny crosses. There were no disease symptoms observed in Gushe, Chamandanda, MK06/0323 and Silira OP crosses. Although Mbundumali was included as a susceptible control, the severity scores were low. This might indicate that the virus infection in plants in the trial occurred late in the season.

Table 3.6 Results of cassava mosaic disease severity and incidence in F1 cassava progenies from open pollinated crosses

Cross progeny	9 MAP		10 MAP		11 MAP		12 MAP	
	Mean Severity	Incidence (%)	Mean Severity	Incidence (%)	Mean Severity	Incidence (%)	Mean Severity	Incidence (%)
Mbundumali OP	1.47	33.30	1.80	40.00	2.20	46.67	3.07	66.67
Kachamba OP	1.47	33.30	1.80	40.00	1.40	40.00	1.67	46.70
Beatrice OP	1.13	6.67	1.13	6.67	1.40	20.00	1.47	20.00
Gushe OP	1.00	0.00	1.00	0.00	1.00	0.00	1.07	20.00
Chamandanda OP	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
MK06/0323 OP	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
Silira OP	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
Mbundumali (Control)	1.20	20.00	1.40	26.70	2.00	46.70	2.60	60.00
Mulola (Control)	1.00	0.0	1.07	6.70	1.13	13.30	1.20	20.00
LSD (0.05)	**0.35**	**18.55**	**0.39**	**18.25**	**0.53**	**22.96**	**0.34**	**19.57**
Grand mean	**1.14**	**10.40**	**1.24**	**13.30**	**1.35**	**18.50**	**1.56**	**24.40**
Sig	*	**	**	**	**	**	**	**

LSD - Least significant difference; Sig - significance level; MAP - months after planting

During the final CMD scoring at 12 MAP, CMD severity was highly significantly different. Mbundumali OP cross had the highest CMD severity (3.07) at 12 MAP. This was followed by the control, Mbundumali (2.60). Kachamba and Beatrice OP crosses were similar with severity scores of 1.67 and 1.47 respectively, but they were significantly different from the rest. The tolerant control (Mulola) was similar to Gushe, Silira, Chamandanda and MK06/0323 OP crosses. Generally, during the entire period of disease scoring Mbundumali OP and Kachamba OP crosses showed higher CMD symptom severity than the rest of the progeny crosses. The high CMD symptom severity in Mundumali and Kachamba OP crosses in this experiment might be attributed to the susceptibility of the maternal genotypes from which these F1 progeny crosses were obtained.

The results show that OP crosses from susceptible maternal genotypes had high CMD symptom severity while those from the tolerant/ moderately resistant maternal parents had low CMD symptom severity except for Beatrice OP which might have received more pollen from susceptible plants than the other F1 progeny crosses. This shows that with regard to CMD tolerance these F1 progeny crosses inherited more traits from the maternal genotypes. This might mean that there was not much insect-pollinator activities to pollinate all the flowers since cassava is almost not wind pollinated because the pollen is sticky. There was an increase in symptom severity each month in almost all progenies from crosses that showed CMD symptoms, except Kachamba OP where the CMD severity decreased at eleven MAP. However the increases were not linear. The decrease in symptom severity in Kachamba OP cross corroborates the finding of Ogbe et al. (2003) in which some genotypes were reported to have lower CMD symptom severity at later stages of sampling; but the decrease observed in this research was minimal and the disease severity increased during the subsequent scoring at twelve MAP. However, more time was needed to ascertain if this was the beginning of recovery from infection of this progeny cross. In terms of CMD resistance, Mbundumali OP and Kachamba OP crosses performed poorly as the disease severity scores were similar to the susceptible control (Mbundumali) during the entire sampling period.

Beatrice OP did not perform well although it was better than Mbundumali and Kachamba OP crosses. Therefore, Mbundumali, Kachamba and Beatrice OP crosses should not be considered for further evaluation of CMD resistance. Figure 3.1 shows CMD severity from nine to twelve MAP compared to the controls. It shows that CMD symptom development can sometimes be linear for some genotypesas observed in Mulola (control) from nine MAP to twelve MAP and Mbundumali (control) from ten MAP to twelve MAP. However, this phenomenon might just be coincidental.
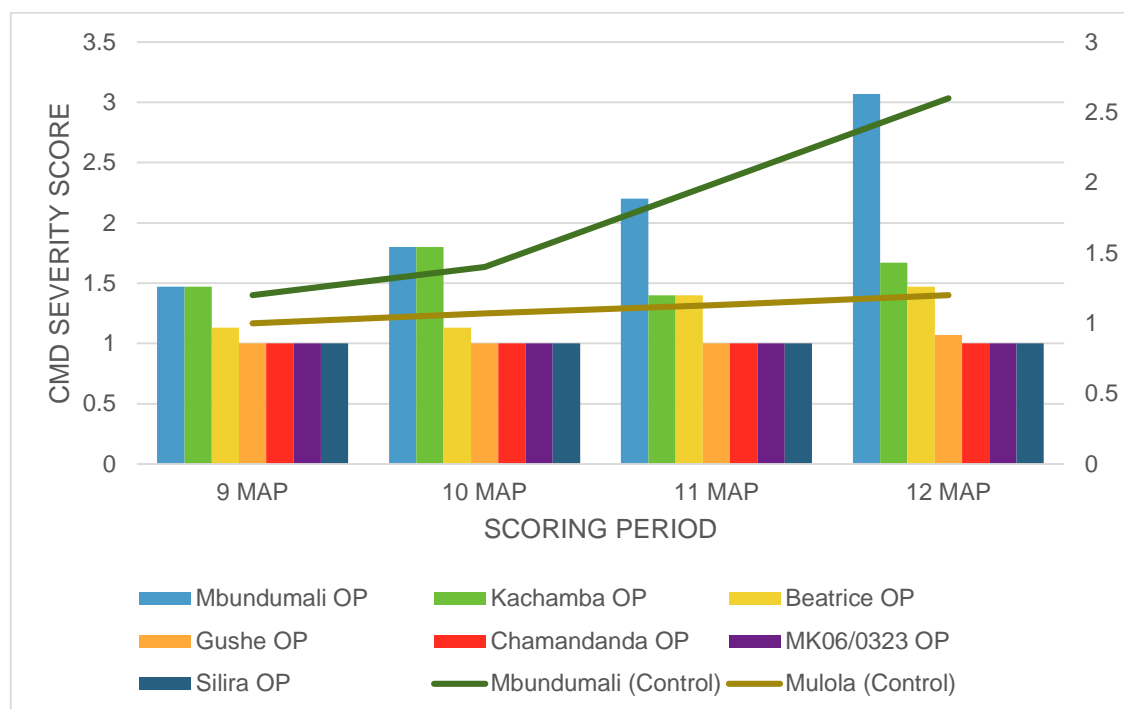
CMD – Cassava Mosaic Disease; OP – Open pollinated; MAP – Months after planting

Figure 3.1 Graph showing cassava mosaic disease severity in F1 cassava progenies from open pollinated crosses. MAP stands for months after planting.

The ANOVA results (Table 3.6) showed that in terms of CMD incidence, Mbundumali, and Kachamba OP crosses were similar to Mbundumali (control) at nine MAP and had disease incidences of 33.30%, 33.30% and 20% respectively. However, they were significantly different from the tolerant control (Mulola) and the rest of the F1 progeny crosses. There was no disease incidence in Gushe, Chamandanda, MK06/0323 and Silira OP crosses. At 10 MAP, no significant difference was observed in Mbundumali OP, Kachamba OP and Mbundumali (control). There was increase in the number of plants showing CMD symptoms in Mbundumali, Kachamba OP crosses and Mbundumali (control) with disease incidences of 40.00%, 40.00%, and 26.70% respectively. There was no disease incidence in Gushe, Chamandanda, MK06/0323 and Silira OP crosses. At eleven MAP, there was no significant differences between Mbundumali OP, Kachamba OP and Mbundumali (control). However, there was an increase in incidence in Mbundumali OP cross and Mbundumali (control) and they had disease incidence of 46.67% which was the highest at this time of sampling. Kachamba and Beatrice OP crosses were not significantly different. However, these progeny crosses were significantly different from the rest of the progeny crosses. There was still no disease incidence in Gushe, Chamandanda, MK06/0323 and

Silira OP crosses at eleven MAP. At 12 MAP, Mbundumali, Kachamba OP crosses and Mbundumali (control) were significantly different from the tolerant control (Mulola) and the rest of the F1 progeny crosses. At this stage of disease scoring, Gushe OP showed some CMD incidence (20%) and was similar to Beatrice OP cross. The susceptible control (Mbundumali) was similar to Mbundumali and Kachamba OP crosses at twelve MAP. The increase in CMD incidence in progeny crosses over time in F1 progeny crosses and controls and the development of disease symptoms at twelve MAP in Gushe OP cross showed that CMD incidence progresses as time goes on until certain period of the year as reported by Fargette et al. (1993) and Fondong et al. (2000).

Gushe, Chamandanda, MK06/0323 and Silira OP progeny crosses were not significantly different from a tolerant control (Mulola) at all sampling intervals. CMD incidence was observed to increase in the F1 progeny crosses and controls that were infected. The incidence results showed that the number of plants in a population of a cassava genotype attacked by CMD may change over time. However, the change may not be linear. The OP crosses that showed high CMD severity (Mbundumali, Kachamba and Beatrice) had high incidence of the disease. There were no foliar symptoms of CBSD in any F1 progenies from OP crosses observed at any interval of sampling. Figure 3.2 shows that CMD incidence can sometimes be linear as observed in Mulola (control) from nine to twelve MAP. However, this phenomenon might just be coincidental in this case as CMD incidence in Mbundumali (control) is not linear.
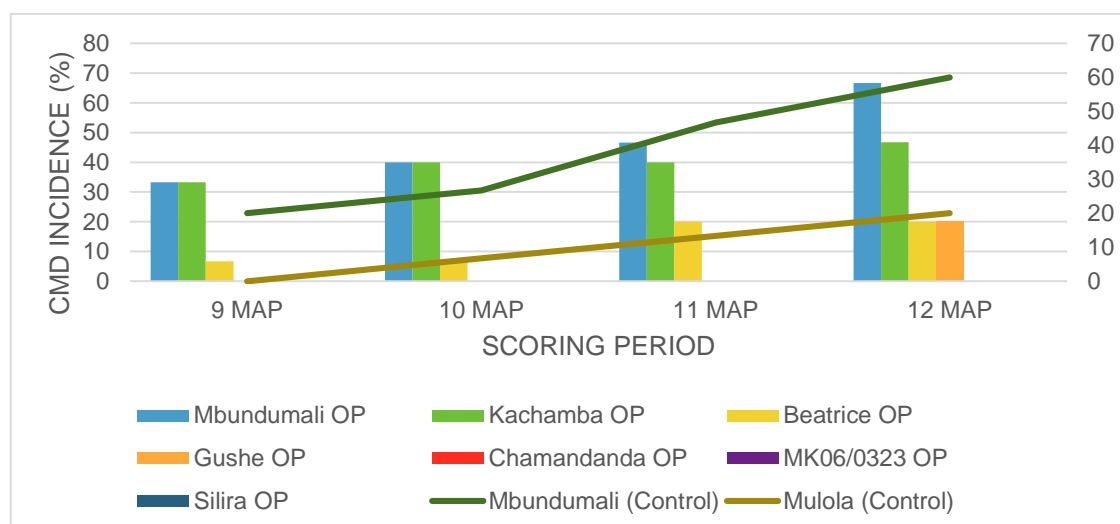
CMD incidence – Percentage plants with cassava mosaic disease symptoms; MAP – Months after planting

Figure 3.2 Graph showing cassava mosaic disease incidence in F1 cassava progenies from open pollinated crosses.

3.3.2 Quantification of *East African cassava mosaic Malawi virus* and *South African cassava mosaic virus* titre in F1 progenies of open pollinated crosses

Both EACMMV and SACMV were detected in all the F1 progeny crosses screened at 9 MAP. This shows that all plants were indeed infected at nine MAP although only some showed disease symptoms. For example Mbundumali OP cross only showed a disease incidence of 33% at nine MAP. The virus quantity was relatively low at nine MAP (Table 3.7). EACMMV and SACMV existed as co-infection in the progenies and controls during the period of the study.

Relative virus quantities were significantly different in the F1 progeny crosses during the sampling period (Table 3.7). Both EACMMV and SACMV increased in Mbundumali OP, Kachamba OP and Mbundumali (control) at 10 and 11 MAP. Mbundumali OP had the virus titre of 785.23 and 290.91 for EACMMV and SACMV respectively which were the highest at ten MAP. Mbundumali (control) and Kachamba OP had increases of both EACMMV and SACMV at ten MAP. However, EACMMV quantities in Mbundumali OP and Mbundumali (control) decreased at eleven MAP while in Kachamba the quantities kept on increasing. The rest of the F1 progeny crosses had low virus quantities during the experimental period with relatively small changes.

There were decreases in virus titre in some progeny crosses in subsequent sampling instead of increasing. For example, SACMV quantities in Mbundumali and Kachamba OP crosses decreased at twelve MAP when compared to the preceding sampling. This phenomenon is not strange as it corroborates the results reported by Ogbe et al. (2003) where a number of genotypes were reported to have lower ACMV concentrations during the subsequent sampling and analysis. Kaweesi (2014) attributed the decrease in virus quantities to the point of not being detected to the virus suppression mechanisms in some genotypes. Generally, Mbundumali, Kachamba OP crosses and Mbundumali (control) had the highest virus quantities.

Table 3.7 Titre for *East African cassava mosaic Malawi virus* and *South African cassava mosaic virus* in F1 cassava progenies from

open pollinated crosses

Progeny	9 MAP		10 MAP		11 MAP		12 MAP	
	EACMMV	SACMV	EACMMV	SACMV	EACMMV	SACMV	EACMMV	SACMV
Mbundumali OP	119.97	13.52	785.23	290.91	658.49	384.09	601.15	311.83
Kachamba OP	13.91	3.95	224.16	146.00	309.65	300.00	270.71	200.91
Beatrice OP	1.00	1.00	3.97	0.56	8.20	1.91	2.03	2.57
Gushe OP	0.01	0.00	0.20	0.10	0.00	0.70	0.13	0.00
Chamandanda OP	0.08	0.07	1.01	0.01	0.02	0.00	0.06	0.00
MK06/0323 OP	0.00	0.00	1.03	0.09	1.03	0.00	0.00	0.00
Silira OP	0.00	0.02	1.11	0.00	0.01	0.00	0.04	0.00
Mbundumali (Control)	10.92	0.95	491.73	100.01	405.72	291.74	720.00	409.55
Mulola (Control)	0.37	1.00	1.77	0.90	1.99	4.15	4.61	2.09
LSD (0.05)	1.77	1.38	150.60	60.25	30.62	44.41	52.73	31.77
Sig	**	**	**	**	**	**	**	**
SD	3.58	1.80	217.60	82.26	212.80	159.00	235.50	156.50

LSD - Least significant difference; Sig - significance level; MAP - months after planting; SD - standard deviation

Figures 3.3 and 3.4 show that the rate of increase of EACMMV and SACV in different genotypes varies. While some genotypes might show rapid increase in EACMMV and SACMV quantities, others show slow or no increase in the quantities. However, the rate of virus accumulation and CMD symptom severity expression over time did not follow a straight line increase/decrease. This might be due to the influence of the environment on the virus and *B. tabaci* and growth activities of the plants (Fargette et al., 1993) especially considering that the experiment was conducted in the field. In Figures 3.3 and 3.4, lines for Mbundumali OP, mbundumali (control) and Kachamba OP are the most conspicuous as they have higher relative virus quantities than the rest. Lines for other progenies lie on the x-axis of the figures due to the relative quantity values. Results show that EACMMV was generally in higher quantities than SACMV as indicated in Figures 3.5 and 3.6 which show smaller threshold cyle (Ct) values of EACMMV than SACMV at 12 MAP. Rubisco had the lowest Ct values as shown in Figure 3.7. The mean Ct values for EACMMV, SACMV and RubisCO from nine MAP to twelve MAP are presented in Appendices 1, 2 and 3. The standard deviations of the technical replicates are presented in Appendices 12, 13, 14 and 15.
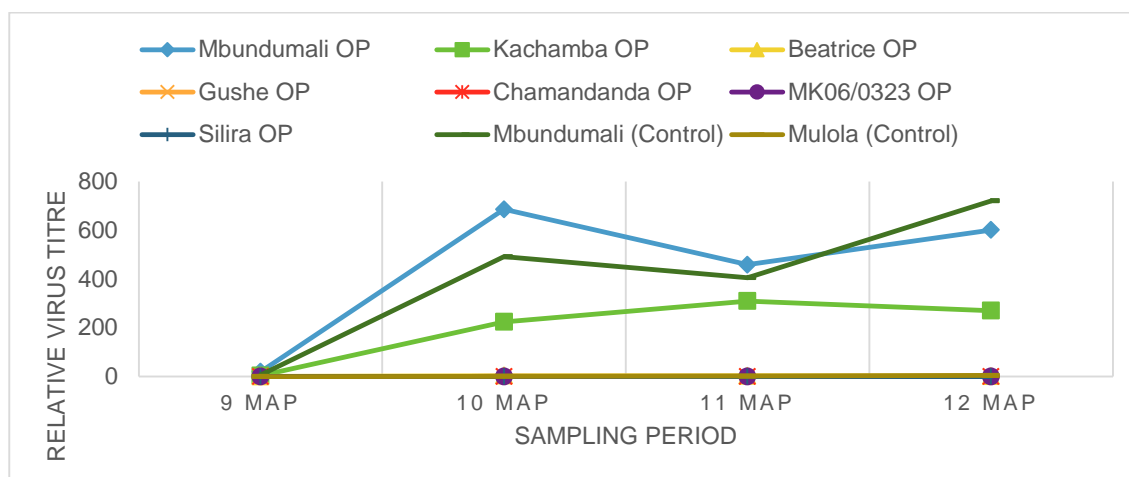
MAP – Months after planting

Figure 3.3 Graph showing accumulation of *East African cassava mosaic Malawi virus* in F1 cassava progenies from open pollinated crosses.
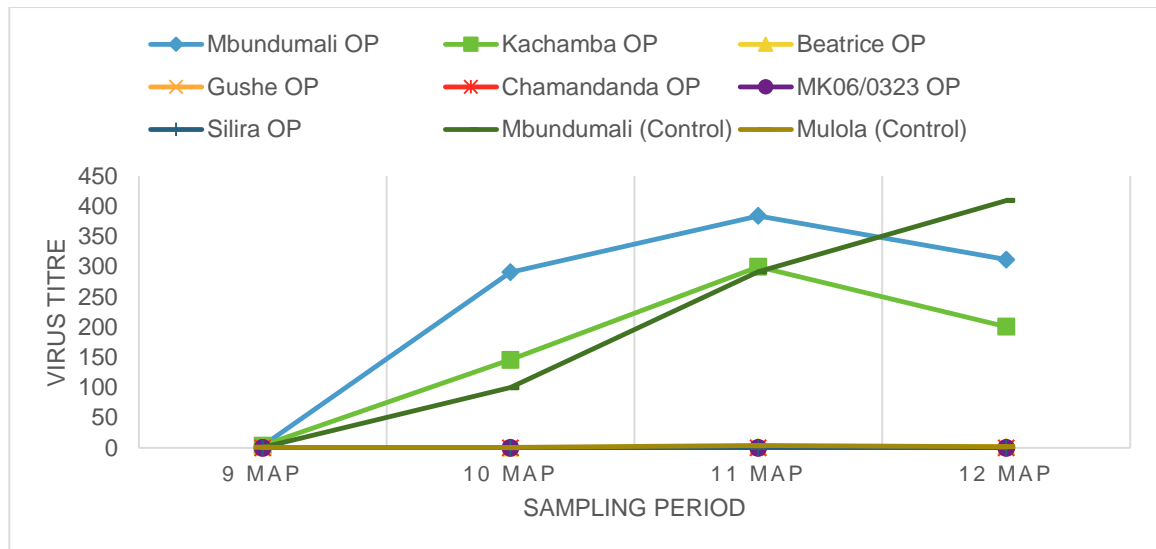
MAP – Months after planting

Figure 3.4 Graph showing accumulation of *South African cassava mosaic virus* in F1 cassava progenies from open pollinated crosses
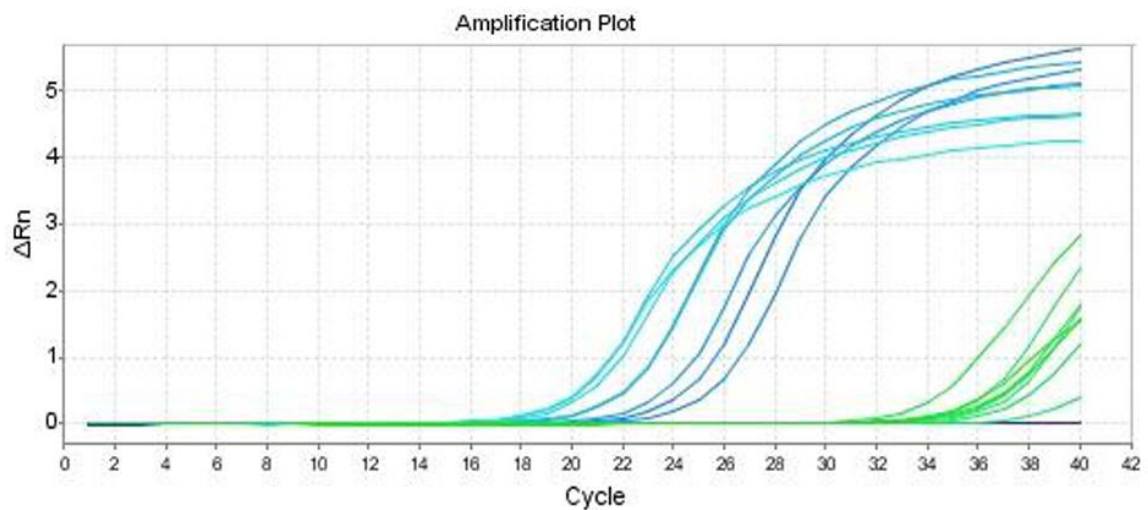
Figure 3.5 Example of amplification plot for *East African cassava mosaic Malawi virus* at Twelve MAP
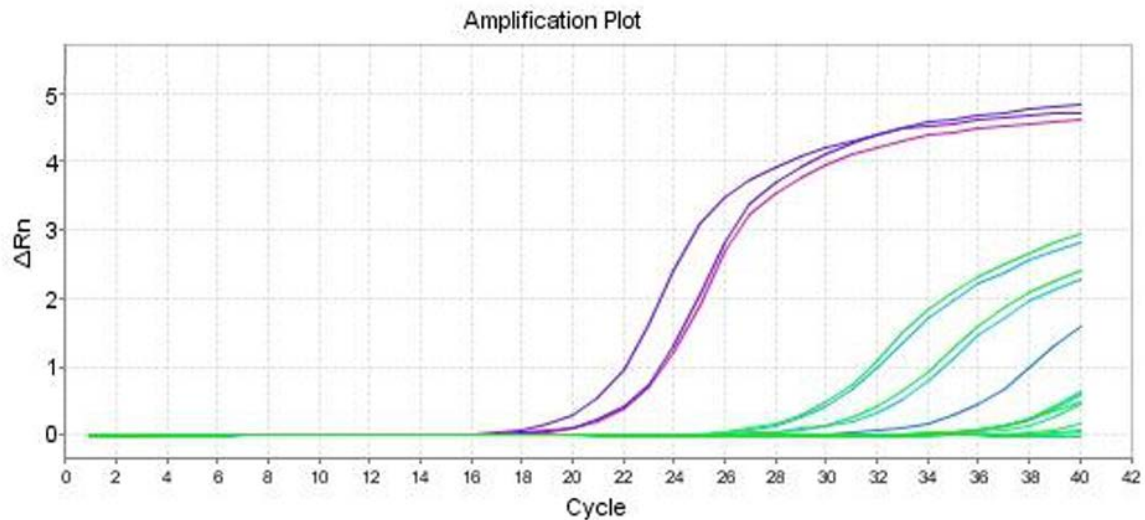
Figure 3.6 Example of amplification plot for *South African cassava mosaic virus* at 12 MAP
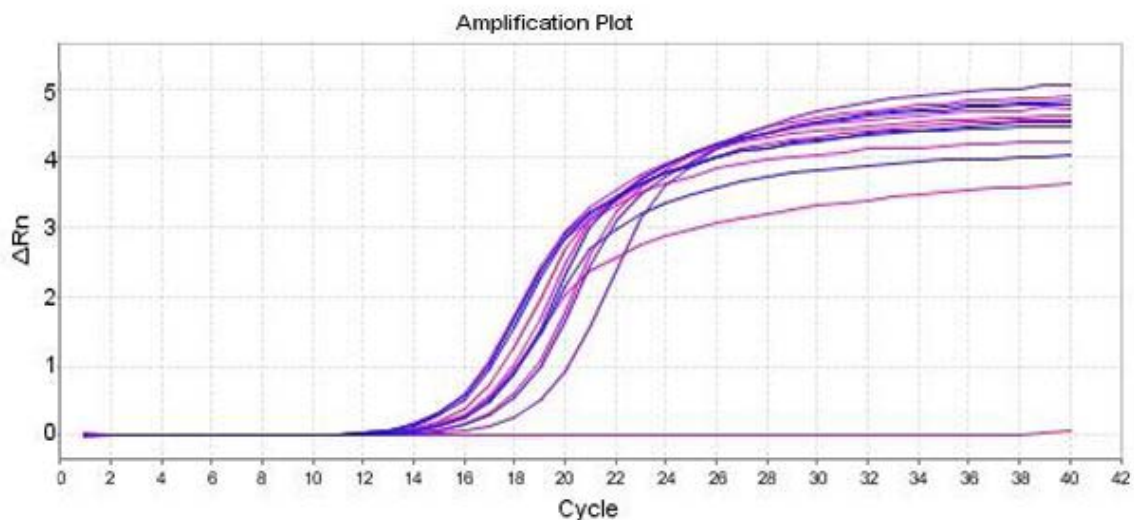
Figure 3.7 Example of amplification plot for reference gene, ribulose-1,5-bisphosphate carboxylase oxygenase at 12 MAP

3.3.3 Comparison between *East African cassava mosaic Malawi virus* titre, *South African cassava mosaic virus* titre, disease symptom severity and incidence in open pollinated crosses

Kachamba, Mbundumali OP crosses and Mbundumali (control) which had higher disease severity scores, had higher EACMMV and SACMV titre than other F1 progeny crosses such as Gushe, Chamandanda, MK06/0323 and Silira OP crosses. Mbundumali OP cross had higher CMD severity and incidence at twelve MAP but both EACMMV and SACMV quantities were less than in the susceptible control (Mbundumali). This shows that a genotype with the highest CMD severity symptoms might not always have highest virus

titre as explained by Ogbe et al. (2003) who found equal virus titre of ACMV in genotypes which showed different degrees of disease symptom expression. The findings in this study suggests that some genotypes may show the same degree of CMD severity while having varying quantities of viruses as observed at 9 MAP in Mbundumali and Kachamba OP crosses. This might suggest that virus replication and symptom expression are controlled by distinct genes in cassava as alluded to by Kaweesi (2014) when working with CBSV and UCBSV.

Table 3.8 shows that there was a significant positive correlation between CMD severity, incidence, EACMMV and SACMV titre at p=0.001. These results on symptom severity and incidence corroborate the findings of Njock and Sama (2015) who found a positive correlation between CMD severity and incidence. Results corroborate the findings reported by Kaweesi et al. (2014) who reported a correlation between virus load and CBSD severity symptoms in cassava plants that showed foliar symptoms. This indicates that cassava genotypes that show high disease incidence are likely to have plants that show severe CMD symptoms. When there is an increase in virus quantity the cassava plants are likely to exhibit severe symptoms. However, CMD severity and virus quantities are not directly proportional. Although Chamandanda, MK06/0323 and Silira OP crosses did not show any foliar disease symptoms, they were infected with viruses even though in low quantities. This observation would assist in emphasising the significance of virus testing especially in cassava seed systems, cassava breeding and plant pathology experiments.

Table 3.8 Spearman rank correlation matrix for cassava mosaic disease severity, incidence, *East African cassava mosaic Malawi virus* (EACMMV) and *South African cassava mosaic virus* (SACMV) titre in F1 cassava progenies from open pollinated crosses

	EACMMV titre	SACMV titre	CMD incidence	CMD severity
EACMMV titre	1.00			
SACMV titre	0.87	1.00		
CMD incidence	0.89	0.88	1.00	
CMD severity	0.90	0.90	0.98	1.00

3.3.4 Cassava brown streak disease severity and incidence in F1 progenies from open pollinated crosses

There were no observable symptoms of CBSD in all F1 progeny crosses as well as the susceptible control (Mulola) during the entire period of study.

3.3.5 Quantification of *Cassava brown streak virus* and *Ugandan cassava brown streak virus* titre in F1 progenies of open pollinated crosses

The plants were screened for CBSV and UCBSV but none of the viruses was detected during the entire experimental period. Figures 3.8, 3.9 and 3.10 show that there was no amplification of the treatment samples. However only positive controls and reference gene amplified. This gives an indication of the reason there was no CBSD symptoms observed in the plants as already stated in section 3.3.4. There might have been low inoculum pressure for CBSD which lead to plants not being infected by CBSD causing viruses.
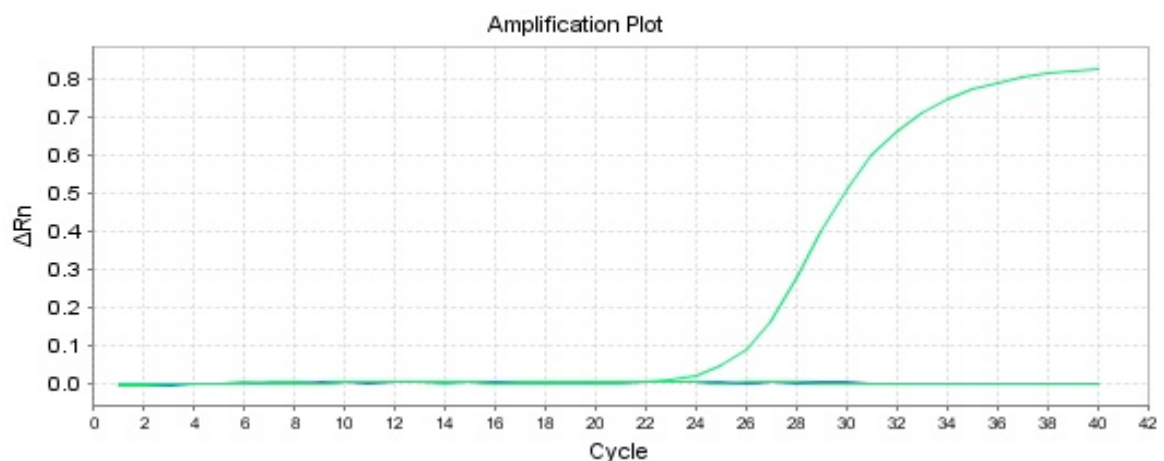
Figure 3.8 Example of amplification plot for positive control sample of *Cassava brown streak virus* at 12 MAP
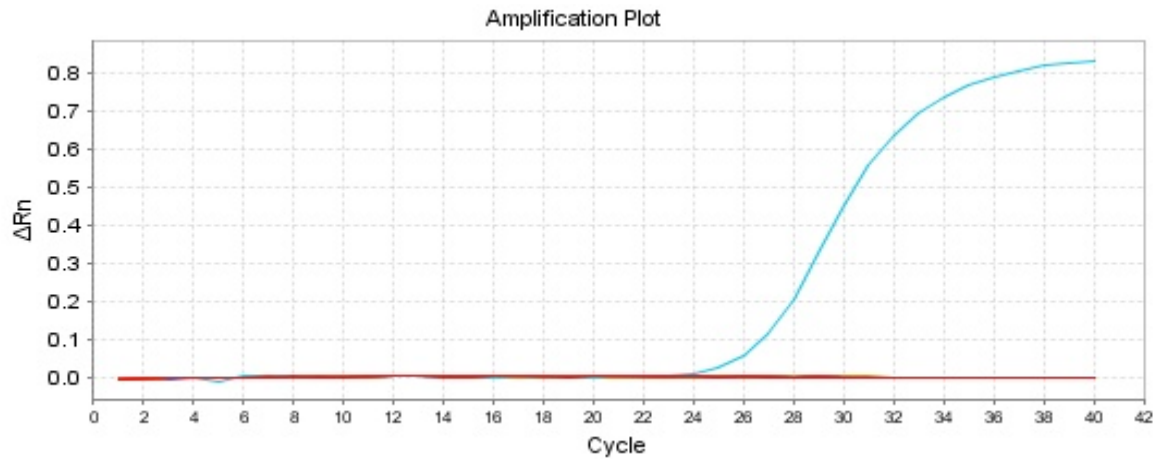
Figure 3.9 Example of amplification plot for positive control sample of *Ugandan cassava brown streak virus* at 12 MAP
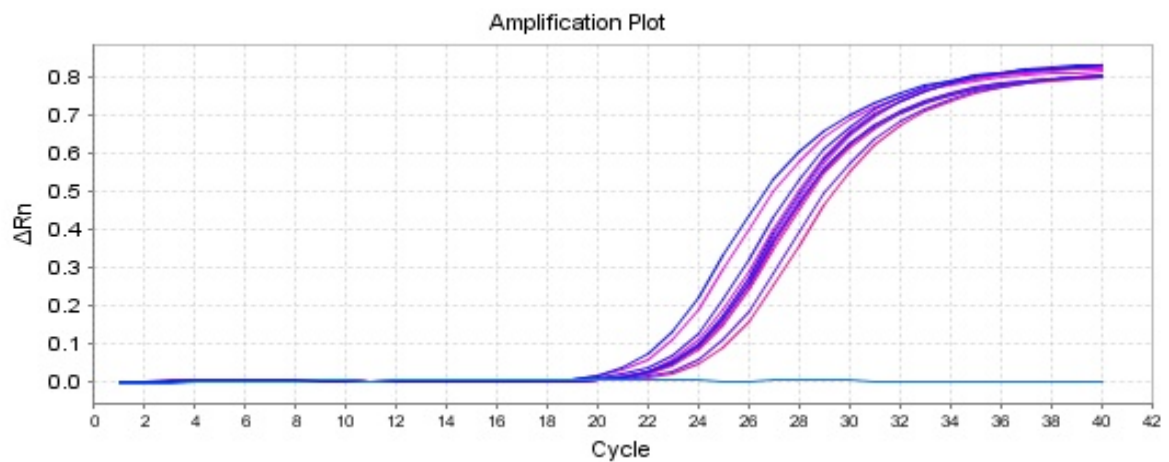
Figure 3.10 Example of amplification plot for reference gene, cytochrome oxidase at 12 MAP

3.4 Conclusions and recommendations

Cassava genotypes have different CMD symptom severity expressions as well as causative virus multiplication rates. Plants of a genotype that exhibit severe disease symptoms are likely to have high virus quantities when analysed in the laboratory. There were correlations between CMD symptom severity, EACMMV and SACMV titre which shows that virus quantity influences foliar symptom severity. However, the rate of virus accumulation and CMD symptom severity expression are neither linear nor exponential. There is a possibility that in a controlled environment, the rate and trend of virus multiplication might be different. CMD severity expression and virus quantities differ depending on the response of the genotype and the rate of activities within the infected genotypes which are also influenced by the environment. There is a possibility that

EACMMV and SACMV quantities may decrease at some point of cassava growth. Therefore, it is necessary to do an investigation on whether the crosses that seem to suppress the viruses do that by complete elimination or by just preventing the virus from accumulating in the leaves which are mainly sampled for laboratory analysis of viruses. Clonal evaluation of OP progenies such as Silira, MK 06/0323, Gushe and Chamandanda with low virus quantities as well as low CMD symptom severity need to be incorporated into the breeding programme for further evaluation. Since Mbundumali is liked due to its sweetness, it is recommended that it should be included in the breeding programme with progenies that showed low virus titre and low disease severity so as to benefit from its sweetness trait while acquiring tolerance to CMD. There is need to re-evaluate these F1 progenies for CBSV and UCBSV for a second season since these viruses were not detected in these progenies during the first growing season, although other experimental trials conducted on the same sites showed CBSD incidence.

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CHAPTER 4

Determining variations in virus titre of cassava mosaic geminiviruses and cassava brown streak viruses in different cassava tissues

4.1 Introduction

Cassava plant is made up of different organs like stems, roots and leaves which are composed of various tissues. Leaves of cassava are arranged spirally on raised nodal portions on the stem. The leaf petiole varies from 5-30 cm in length (Onwueme, 1978). The lower mesophyll surface of cassava leaf is populated with epidermal cells while the upper surface is fairly smooth (Angelov et al., 1993). Leaves have distinct green bundle sheath cells, with small, thin walled cells, which are partially separated below the palisade cells; these cells transport the photosynthates, apart from performing photosynthesis (Alves, 2002). The growth and productivity of cassava depend mostly on leaf canopy capacity to intercept solar radiation during most of the growth cycle and on leaf photosynthetic potential and performance under prevailing field conditions (De Tafur et al., 1997; El-Sharkawy, 2004), therefore leaf distortion by virus diseases such as CMD and CBSD is likely to negatively affect the plant performance.

Cassava stems, which are used for propagation by farmers (Alves, 2002) are known to transmit CMD and CBSD if infected planting materials are used. Chitedze Research Station distributes virus tested materials to farmers based on leaf diagnosis only. Furthermore, the distribution of the viruses causing CMD and CBSD within the stem is not explicitly known.

The mature storage root of a cassava plant has three distinct tissues: periderm, cortex and parenchyma (Onwueme, 1978; Alves, 2002). The parenchyma is the edible portion of the fresh root and comprises approximately 85% of total weight (Wheatley and Chuzel, 1993). The parenchyma consists of xylem vessels which are radially distributed in a matrix of starch containing cells (Wheatley and Chuzel, 1993). The peel layer, which is comprised of sclerenchyma, cortical parenchyma and phloem, constitutes 10–20% of root weight (Onwueme, 1978). The periderm which makes up 3% of total root weight, is a thin layer made of a few cells thick and becomes the outermost portions. Cell growth in these cells usually slow down as the plant matures. Since the root is composed of anatomically varying tissues, it would be expected that the quantity of molecular particles such as viruses would be different. Compounds like cyanide are reported to be distributed within the whole

cassava plant except seeds, and enzyme molecules such as, hydroxynitrile lyse are tissue specific (White et al., 1998; Santana et al., 2002).

The type of tissues and cells of a plant determines the tissue tropism of the virus (Morra and Petty, 2000). Different viruses show different tissue tropism. For example, geminivirus, *Indian cassava mosaic virus* (ICMV) has been reported to accumulate in phloem. ACMV and *Tomato golden mosaic virus* (TGMV) have been reported to invade apart from the phloem, the spongy parenchyma, palisade and epidermal cells (Wege et al., 2001). Plant viruses move in plants from cell-to-cell through cytoplasmic connections called plasmodesmata which depends on a functional cell-to-cell movement of proteins and other gene products (Hull, 2009). Differences in proteins and other gene products among plants may thus affect the distribution of viruses. There is the possibility of a virus replicating in a particular cell but failing to move to adjacent cells in a situation called sublimal infection (Rothenstein et al., 2007; Hull, 2009). A gene product, nuclear shuttle protein, for BV1 on DNA-B in geminiviruses is responsible for transporting viral DNA from nucleus to cytoplasm, which facilitates the movement of viruses from cell-to-cell (Abraham, 2012); hence from tissue-to-tissue. Replication of a plant virus in a cell depends on the activities of the host (Nagar et al., 2002). In reaction to virus invasion in plant cells, plants have gene silencing defence systems against foreign nucleic acids (Hull, 2009). In geminiviruses, the gene product for AV2 in DNA-A is involved in virus accumulation and symptom development (Padidam et al., 1992). Genetic constitution of a plant is determining to whether a virus will replicate and distribute in it, although viruses themselves have mechanisms of overcoming plant defence responses.

In the development of cassava varieties, breeders have to develop varieties that are distinct in a number of ways that include disease resistance, drought tolerance and yield. The response of plants to pathogens, let alone viruses, as mentioned above, would depend on the genetic constitution of a plant to allow the virus to move from one point to the next. As such, genetically distinct cassava varieties differ in their ability to prevent virus distribution within a plant. Ogwok et al. (2015) reported high accumulation of CBSV titre in roots than aerial organs in resistant genotypes. However, there were variations in virus titre of each organ in susceptible genotypes.

If plants have a mechanism of confining the virus to a particular part of the plant or plant organ, it is easy for an investigator to make erroneous conclusions about the presence of viruses in plants and resistance of genotypes, particularly if a less sensitive diagnosis technique is used. Cassava genotypes which accommodate the highest virus quantities in

the leaves would have high disease incidence and severity as whitefly vectors would be easily in contact with large amount of viral particles. These would not be regarded as resistant. It would be presumed that resistant/tolerant genotypes would not contain high virus quantity in leaves in relation to CBSV (Ogwok et al., 2015). Therefore, research on the quantities of the CMG and CBSV in cassava plants and organs will assist to elucidate the relationship between plant tissue and virus distribution in plants. This will in return help to identify genotypes that would restrict the spread of CMD and CBSD causing viruses by limiting the contact between whiteflies and viruses. This is a good trait for disease resistance breeding. Further more this research will help to improve cassava virus diseases diagnostics. The aim of this study was therefore to determine variations in virus titre in different cassava tissues.

4.2 Materials and methods

4.2.1 Planting materials and experimental site

The study was carried out during the 2014/2015 growing season. Plants grown in a seed multiplication block in which plants were spaced at 1 m x 1 m at Chitedze Research Station were used for determining the variations of virus titre of different species of CMG and CBSV in different cassava tissues. Chitedze Research Station is located near the capital city of Malawi, Lilongwe, which is located 13^0 59´S and 33^0 38´E at 1146 m above sea level. Lilongwe plain is located on the mid-altitude plateau of Central Africa. The cultivars used were Mbundumali, Mulola and Kalawe. Cassava cultivars showing foliar symptoms of CMD and CBSD were randomly sampled. For each cultivar, seven plants were analysed for SACMV, EACMMV, CBSV and UCBSV titre. Cassava plants were sampled at 12 MAP, the time farmers start harvesting the crop for consumption or as source of planting material. Tissue sampling was done and the following tissue types were collected from plants showing disease symptoms: leaves (first fully open leaf, fifth fully open leaf and tenth fully open leaf), stem bark (5 cm from the top most part the plant, the middle of the stem, and 5 cm from the roots), root parenchyma (starchy fresh), and root cortex (peels). After sampling, samples were taken to the Biotechnology laboratory at Chitedze Research Station for analysis. The tolerance/susceptibility to CMD and CBSD of the cultivars used are described in Table 4.1.

Table 4.1 Description of genotypes from which tissue samples were taken

Variety	Tolerance/susceptibility
Mbundumali	Tolerant to CBSD and CGM but susceptible to CMD
Kalawe	Moderately resistant to CMD and CBSD
Mulola	Tolerant to CMD, CM and CGM

CBSD – Cassava Brown Streak Disease; CGM – Cassava Green Mite; CM – Cassava Mealy Bug; CMD – Cassava Mosaic Disease

Haggblade and Zulu (2003); USAID (2011); Alene et al. (2013)

4.2.2 Extraction of total nucleic acids

TNA were extracted from 100 mg ground fresh tissue samples according to the procedure described in section 3.2.2.

4.2.3 Quantification of *cassava brown streak virus* and *Ugandan cassava brown streak virus*

Quantification of the CBSV and UCBSV titre in cassava varieties was done using TaqMan chemistry. COX was used as internal control for data normalisation as reference gene. Probes and their complementary primers were used as described in section 3.2.7.2. Sterile distilled water was used as a no template control, and there were two positive controls from samples that previously showed amplifications of CBSV and UCBSV. ROX was used as a passive reference. The RNA sample of the 5th fully open leaf of Mulola was used as calibrator sample. The qPCR reactions contained 11.46 µl sterile distilled water, 2.0 µl of 10x PCR buffer, 2.8 µl of 25 mM $MgCl_2$, 1.0 µl of 10mM dNTPs, 0.8 µl of 7.5 mM reverse and forward primers, 0.4 µl of 5 µM probe, 0.4 µl of 15 µM ROX solution, 0.1 µl of 5U µl^{-1} Taq DNA polymerase, 0.04 µl of 200U µl^{-1} MMLV reverse transcriptase and 1.0 µl of RNA template in a final volume of 20 µl. The mixture was dispensed into Step One^{TM} Real – Time PCR system (Applied Biosystems) with 48 reaction plates. The qPCR plates were sealed using adhesive PCR seals to provide protection against evaporation and dispersion. The ΔΔCt method was used to determine relative virus titre as described in section 3.2.7. Thermal cycling conditions were performed as described in section 3.2.7. Two technical replicates were used for each reaction. The experiment had three biological replicates. Due to the limitation of the number of wells, the biological replicates were performed on separate qPCR plates and an inter-run calibrator sample was used.

4.2.4 Quantification of *South African cassava mosaic virus* and *East African cassava mosaic Malawi virus*

Quantification of SACMV and EACMMV titre was done using SYBR Green I protocol using step one real-time PCR system as described in section 3.2.7. The DNA sample of the 5th fully open leaf of Mulola was used as calibrator sample. Two technical replicates were used for each reaction. The experiment had three biological replicates.. Due to the limitation of the number of wells, the biological replicates were performed on separate qPCR plates and an inter-run calibrator sample was used.

4.3 Data analysis

Relative virus titre of CBSV, UCBSV, EACMMV and SACMV were subjected to ANOVA using GenStat 17.1 computer software (Payne et al., 2014). The virus titre for co-infections were correlated using Spearman rank correlation matrix.

4.4 Results and discussion

Co-infection of CBSV and UCBSV was observed in six plants of Mbundumali and Mulola. There was one plant with single-infection of CBSV in Mbundumali, seven plants with single-infection of UCBSV in Kalawe and one plant with single-infection of CBSV in Mulola. CBSV and UCBSV relative quantities were significantly different in various tissues of the genotypes used in this study (Table 4.2). In co-infection of Mbundumali, CBSV titre was highest in root parenchyma (23.40), followed by tenth fully opened leaves (5.80), stem bark (5 cm from the top) (3.10) and stem bark (5 cm from the roots) (2.10) respectively. The first fully opened leaf had the lowest CBSV titre in co-infection (1.40). UCBSV titre in Mbundumali co-infection was highest in root parenchyma (4.60), followed by tenth fully open leaf (3.75) and stem bark (5 cm from the top) (2.60). The first and fifth fully open leaves had the lowest virus titre of 1.07 and 1.12 respectively.

High CBSV and UCBSV titre in root parenchyma in Mbundumali genotype was in agreement with findings of Ogwok et al. (2015). However, there were some differences between these results and results reported by Ogwok et al. (2015) especially on the trend of accumulation of the viruses in certain tissues. This might have been due to differences in the genotypes used, the time at which sampling was done, the season in which the experiments were conducted and locations of the experiments. These findings, differed from a study on potato (*Solanum tuberosam*) by Kogovšek et al. (2011) where low quantities of an RNA virus, *Potato virus Y* (PVY), were detected in potato tuber tissues. This might be so because cassava and potato are botanically two different plants.

Table 4.2 Titre for *Cassava brown streak virus* and *Ugandan cassava brown streak virus* in various tissues of three cassava genotypes

Tissue	Co-infection				Single-infection					
	Mbundumali		Mulola		Mbundumali		Mulola		Kalawe	
	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV	UCBSV
1st fully open leaf	1.40	1.07	0.97	0.73	0.99	0.00	0.82	0.00	0.00	2.52
5th fully open leaf	1.80	1.12	1.00	1.00	5.10	0.00	1.00	0.00	0.00	4.72
10th fully open leaf	5.80	3.75	2.27	1.29	5.03	0.00	1.62	0.00	0.00	2.65
Stem bark (5 cm from top)	3.10	2.60	1.60	1.39	4.44	0.00	2.05	0.00	0.00	0.77
Stem bark (middle)	2.00	1.56	1.23	1.09	2.97	0.00	1.39	0.00	0.00	0.22
Stem bark (5 cm from roots)	2.10	1.07	1.05	0.56	1.07	0.00	0.89	0.00	0.00	0.11
Root parenchyma (Non-necrotic)	23.40	4.60	1.53	1.07	4.95	0.00	1.01	0.00	0.00	0.40
Root cortex (Peel)	2.00	0.06	0.49	0.43	1.05	0.00	0.91	0.00	0.00	0.10
LSD (0.05)	0.73	0.36	0.52	0.29	0.62		0.30			0.72
Sig	**	**	**	**	**		**			**
SD	7.10	1.50	0.64	0.39	1.86		0.44			1.72

LSD - least significant difference; Sig - significance level; SD - standard deviation

Furthermore, PVY is confined to phloem, but it has been reported in xylem parenchyma in *Nicotiana tabacum* (Ryang et al., 2004).In single-infection, Mbundumali had the highest CBSV titre in the fifth fully open leaf (5.10), followed by the tenth fully open leaf (5.03), root parenchyma (4.95), stem bark (5 cm from the top) (4.44) and middle stem bark (2.97). The first fully open leaf (0.99), stem bark (5 cm from the roots) (1.07) and the root peel (1.05) had the lowest CBSV titre. There was no single-infection of UCBSV in Mbundumali. High accumulation of CBSV in the fifth and tenth fully open leaves showed that CBSV accumulation might not consistently be high in a single cassava tissue in this case root parenchyma which showed highest CBSV and UCBSV accumulation in co-infection. However, due to inconsistencies in the numbers of plants showing co-infection and single-infection, the experiment did not conclusively compare the virus titre in various tissues when there is co-infection and single-infection.

In co-infected plants, Mulola had the highest CBSV titre in the tenth fully opened leaf (2.27). UCBSV titre in Mulola was highest in stem bark (5 cm from the top) (1.39). This was followed by the tenth fully open leaf (1.29), stem bark (middle) (1.09), root parenchyma (1.07) and the fifth fully open leaf (1.00). Root cortex had the lowest virus titre (0.43). In single-infection, Mulola had the highest CBSV titre in the stem bark (5 cm from the top) (2.05); followed by the tenth fully open leaf (1.62) and middle stem bark (1.39). The first fully open leaf, stem bark (5 cm from the roots) and root cortex had the lowest CBSV titre of 0.82, 0.89 and 0.91 respectively. There was no single-infection of UCBSV detected in Mulola.

There was a significant positive correlation of 0.75 (at p=0.05) and 0.88 (at p=0.001) between CBSV and UCBSV titres of co-infection in Mbundumali and Mulola genotypes respectively as shown in Tables 4.3 and 4.4. This suggests that an increase in CBSV or UCBSV viral particles in one tissue is likely to result in increases of the virus in other tissues as well but they are not directly proportional. It would be thought that since both CBSV and UCBSV are potyviruses, they would have equal quantities in the same co-infected plants. However, in genotypes that showed co-infection of CBSV and UCBSV, the virus quantities of CBSV were relatively higher than those of UCBSV as shown in Figures 4.1, 4.2 and 4.3. This is in agreement with the findings of Mascia et al. (2010) who reported varying virus quantities of potyviruses, *Cucumber mosaic virus* and *Potato virus Y* in mixed-infections of tomato.

Table 4.3 Spearman rank correlation matrix for *Cassava brown streak virus* and *Ugandan cassava brown streak virus* titre in plant tissue of Mbundumali

	CBSV titre	UCBSV titre
CBSV titre	1.00	
UCBSV titre	0.75	1.00

Table 4.4 Spearman rank correlation matrix for *Cassava brown streak virus* and *Ugandan cassava brown streak virus* titre in plant tissue of Mulola

	CBSV titre	UCBSV titre
CBSV titre	1	
UCBSV titre	0.88	1

There were no co-infections or CBSV single-infection observed in Kalawe; but UCBSV was observed in the samples analysed. In single-infection of UCBSV, Kalawe had the highest virus titre in the fifth fully open leaf (4.72); followed by the tenth fully open leaf (2.65) and the first leaf (2.52). The root cortex, stem bark (5 cm from the roots), middle stem bark and the root parenchyma had the lowest virus titre of 0.10, 0.11, 0.22 and 0.40 respectively. These results corroborate the findings of Ogwok et al. (2015) who reported no particular pattern for UCBSV accumulation in tissues of some cassava genotypes. The mean Ct values for CBSV, UCBSV and COX in Mbundumali, Mulola and Kalawe are presented in Appendices 4, 5, 6, 7 and 8.
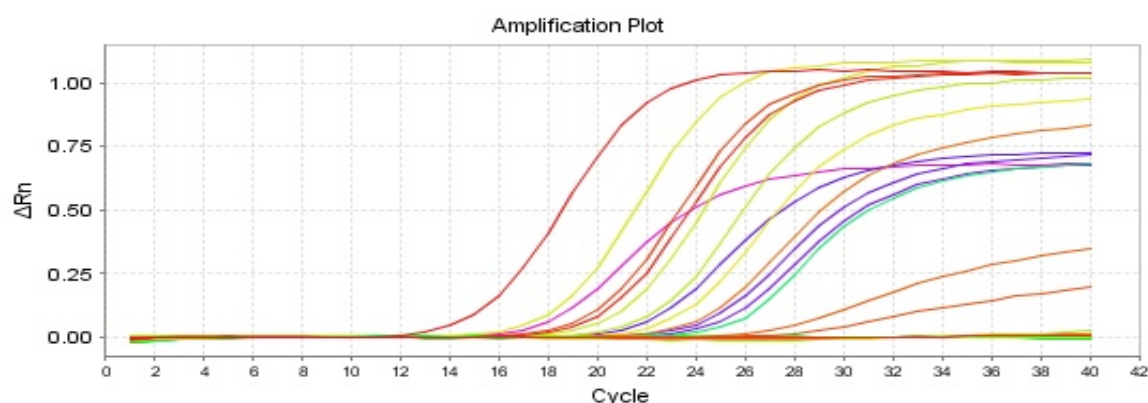
Figure 4.1 Example of amplification plot for *Cassava brown streak virus* in co-infected Mbundumali plant tissue
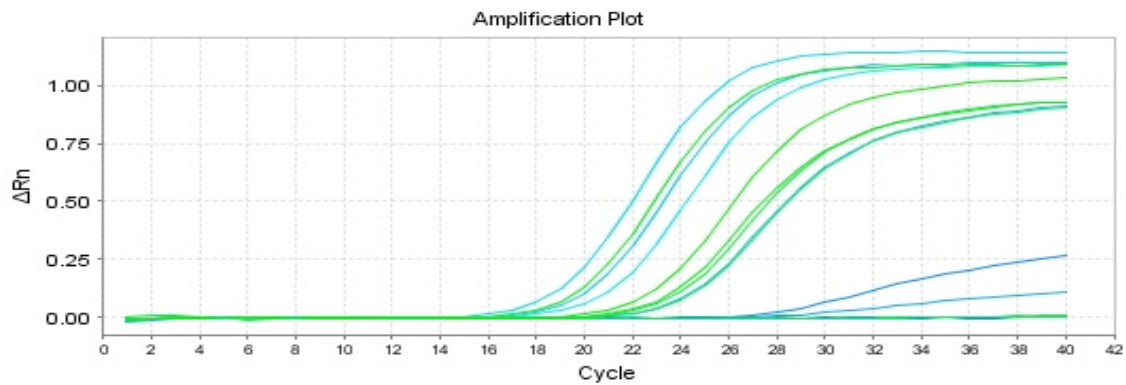
Figure 4.2 Example of amplification plot for *Ugandan cassava brown streak virus* in co-infected Mbundumali plant tissue
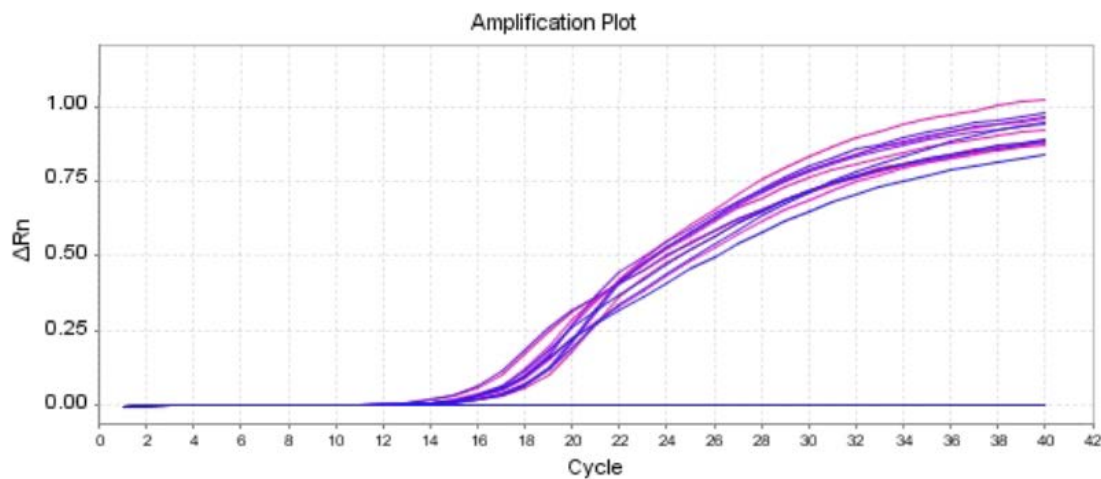
Figure 4.3 Example of amplification plot for cytochrome oxidase in Mbundumali plant tissue

Table 4.5 shows that Mbundumali had the highest EACMMV titre in the first fully open leaves (124.90) followed by the stem bark (5 cm from the top) (17.27), the tenth fully open leaves (13.11) and stem bark (middle) (10.16).

The root cortex had the least EACMMV titre. SACMV titre was highest in the first fully open leaves with a virus titre of 16.01. It was followed by the tenth fully open leaves and the stem bark (5 cm from the top) with a virus titre of 13.00 and 9.11 respectively. The fifth fully open leaf and root cortex had the lowest virus quantities with titres of 1.94 and 1.09 respectively.

In Kalawe, EACMMV quantities were highest in the first fully open leaves with virus titre of 17.33. This was followed by the fifth fully open leaves (10.07). The stem bark (5 cm from

the roots), root parenchyma and root cortex had the lowest EACMMV quantities of 2.01, 1.73 and 1.06 respectively. SACMV quantities were highest in the first fully open leaves (9.97). This was followed by the stem bark (5 cm from the top). The stem bark (5 cm from the roots), root parenchyma and root cortex had the lowest SACMV quantities of 1.40, 1.86 and 0.00 respectively.

In Mulola, EACMMV quantities were highest in the fifth fully open leaves (1.00). This was followed by the tenth fully open leaves and the first fully open with virus titres of 0.91 and 0.59 respectively. SACMV quantities were highest in the fifth fully open leaves (1.00). This was followed by the tenth fully open leaves with virus titre of 0.32. The stem bark had considerably lower quantities of SACMV than the leaves. High quantities of SACMV in some of the leaves and the stem bark (5 cm from the top) might be attributed to growth activeness of these tissues. However, the detection of high virus quantities in root parenchyma in Mulola indicates that SACMV can also be distributed to the roots. Sadeghi et al. (2000) attributed high *barley yellow dwarf virus* (BYDV) replication in leaves of barley to rapid replication of BYDV in phloem cells of growing tissue in barley.

There were significant positive correlations of 0.801, 0.673 and 0.833 between EACMMV and SACMV titre in Mbundumali, Mulola and Kalawe respectively as shown in Table 4.6, 4.7 and 4.8 with probabilities of 0.004, 0.02 and 0.003. This suggests that the quantity of one virus increases with an increase in the other. There is possibility that if conditions are favourable for the increase on one virus, the other virus will also increase. These virus species might be replicated and translocated simultaneously in cassava plants although not with equal rates leading to positive correlation. However, just like in CBSVs, the positive correlations did not imply that there was direct proportionality between EACMMV and SACMV. The presence of EACMMV and SACMV in stem barks corroborates the findings of Njock and Ndip (2007) who detected the presence of a *geminivirus*, ACMV in stems using enzyme-linked immunosorbent assay (ELISA). Just like other geminiviruses, the movement of photo-assimilates to growing parts of the plant might be a way that these viruses are transported, as explained by Ghoshroy et al. (1997), or since in the active growing parts of a plant, cell replication/differentiation is high, these viruses might use the DNA synthesis machinery of the host to replicate in those tissues (Hull, 2009) as observed in a *geminivirus*, *Tomato golden mosaic virus* (TGMV), which replicates in differentiated plant cells using the host DNA synthesis mechanisms (Nagar et al., 2002).

Table 4.5 Titre for *East African cassava mosaic Malawi virus* and *South African cassava mosaic virus* in various tissues of three cassava genotypes

Tissue	Mbundumali		Mulola		Kalawe	
	EACMMV	SACMV	EACMMV	SACMV	EACMMV	SACMV
1st fully open leaf	124.90	16.01	0.59	0.21	17.33	9.97
5th fully open leaf	3.07	1.94	1.00	1.00	10.07	2.71
10th fully open leaf	13.11	13.00	0.91	0.32	3.91	2.59
Stem bark (5cm from top)	17.27	9.11	0.25	0.02	3.64	3.31
Stem bark (middle)	10.16	3.09	0.01	0.04	3.66	2.05
Stem bark (5 cm from roots)	4.11	0.17	0.00	0.01	2.01	1.40
Root parenchyma (Non-necrotic)	4.00	1.50	0.31	0.02	1.73	1.06
Root cortex (Peel)	1.23	1.09	0.06	0.01	1.06	0.00
LSD (0.05)	30.41	4.53	0.31	0.32	3.24	2.28
Sig	**	**	**	**	**	**
SD	48.31	7.41	0.50	0.45	6.34	3.84

LSD= least significant difference; Sig= significance level; SD= standard deviation

Table 4.6 Spearman rank correlation matrix for _East African cassava mosaic Malawi virus_ and _South African cassava mosaic virus_ titre in plant tissue of Mundumali

	EACMMV titre	SACMV titre
EACMMV titre	1.00	
SACMV titre	0.801	1.00

Table 4.7 Spearman rank correlation matrix for _East African cassava mosaic Malawi virus_ and _South African cassava mosaic virus_ titre in plant tissue of Mulola

	EACMMV titre	SACMV titre
EACMMV titre	1.00	
SACMV titre	0.673	1.00

Table 4.8 Spearman rank correlation matrix for _East African cassava mosaic Malawi virus_ and _South African cassava mosaic virus_ titre in plant tissue of Kalawe

	EACMMV titre	SACMV titre
EACMMV titre	1.00	
SACMV titre	0.833	1.00

Generally, the results observed in this research show that EACMMV was in higher quantities than SACMV in most of the tissues of all the genotypes used. This experiment relied on field infection, therefore there is possibility that plants were infected at different times. Some plants might have been infected earlier than others. This might have had an influence on the tissue distribution of the viruses and the virus quantity in different tissue. There is a possibility that in a controlled environment, the results might not exactly be the same as these due to the reasons already mentioned. As shown in Figures 4.4 and 4.5, EACMMV had small Ct values as compared to SACMV in Mbundumali co-infected plant. Rubisco had the smallest Ct values as shown in Figure 4.6. The mean Ct values for EACMMV, SACMV and RubisCO in Mbundumali, Mulola and Kalawe are presented in Appendices 9, 10 and 11.
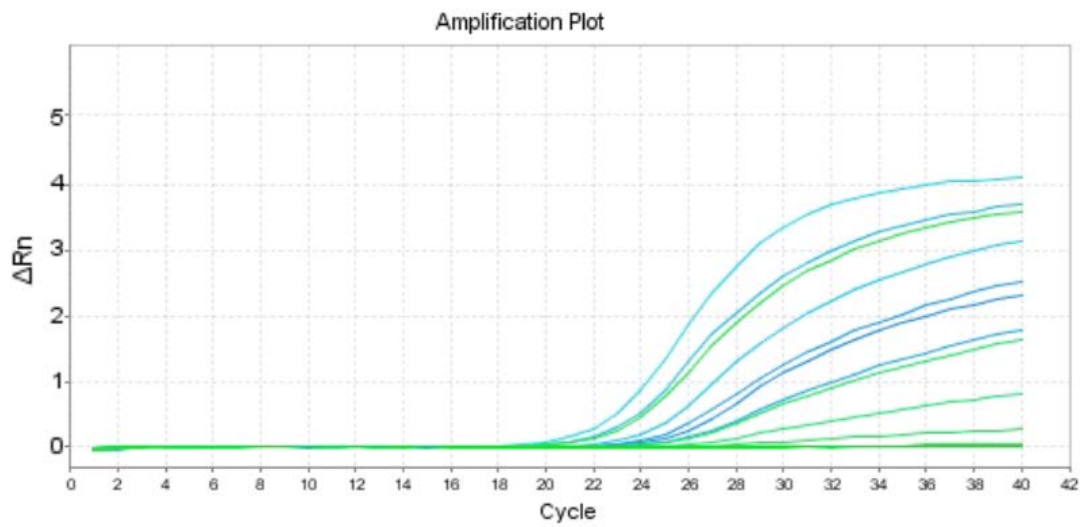
Figure 4.4 Example of amplification plot for *East African cassava mosaic Malawi virus* in Mbundumali plant tissue
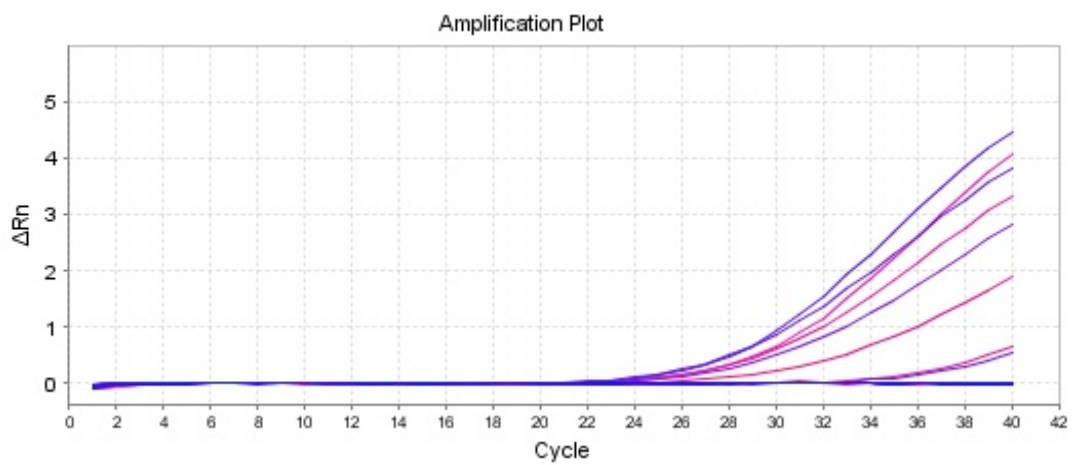
Figure 4.5 Example of amplification plot for *South African cassava mosaic virus* in Mbundumali plant tissue
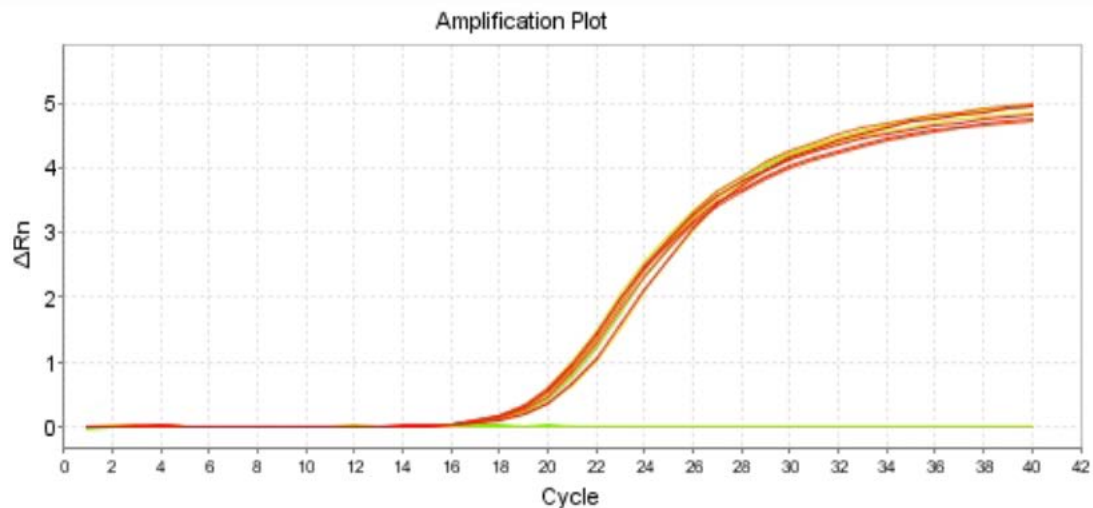
Figure 4.6 Example of amplification plot for reference gene, ribulose-1,5-bisphosphate carboxylase oxygenase in Mbundumali

4.5 Conclusions and recommendations

CBSV, UCBSV and SACMV were detected in the tissue samples analysed. Varying quantities of CBSV and UCBSV in different tissues of the same plants show that these viruses are not uniformly distributed. Generally, the trend of CBSV and UCBSV accumulation in plant tissues is similar in both co-infected and single infected plants. However, the quantities may vary depending on the genotype used. High CBSV and UCBSV titre in root parenchyma tissue of Mbundumali might be attributed to the translocation of the viral particles to these tissues through vascular bundles. However, individual plants showed different trends when analysed.

EACMMV and SACMV accumulated more in the leaves especially the first fully open. Stem bark had a considerable amount of the virus. These are actively growing parts of a plant. However, the quantities may vary based on the genotype and there is a possibility that other tissues could have a high quantity of the viruses, as seen in the results. The stem bark contains phloem that carries plant assimilates to other parts of a plant, therefore it would be expected that these EACMMV and SACMV are carried along.

Cassava disease diagnosis and studies on cassava seed systems and genotype resistance/susceptibility based on CBSV, UCBSV, EACMMV and SACMV quantification will yield better results if cassava plant tissue analysis is done using different tissues which will be analysed concurrently since it has been observed that the distribution of these viruses is uneven. However, leaves are the most reliable tissue to use in disease diagnosis as they usually contain detectable amounts of viruses. There is need to do a study to

determine the virus quantities in different cassava tissues at different time intervals since the distribution of a virus increases the virus availability in plants for vector transmission (Mascia et al. 2010). Therefore, there is a possibility that cassava genotypes which accommodate the highest virus quantities in the leaves would have high disease incidence and severity as whitefly vectors would be easily in contact with large amount of viral particles. However, this needs to be verified because this study was not designed to elucidate this relationship. Since this experiment has not explicitly compared whether co-infection or single infection affect virus accumulation in different tissues of cassava plants, there is a need for further research in a controlled environment that will involve inoculation of the viruses in cassava plants to make sure that equal number of plants with co-infections and single-infections of the viruses analysed in this research are compared. Based on the results obtained in this study, it would be concluded that the first fully open leaves need to be sampled for SACMV and EACMMV diagnosis. The third fully open leaves need to be sampled when doing CBSV and UCBSV diagnosis. If practicable, root parenchyma sampling will be necessary as it has been observed that they accommodate the largest amount of CBSV and UCBSV.

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CHAPTER 5

Detection of cassava mosaic geminiviruses and cassava brown streak viruses in cassava seedlings grown from true seeds obtained from diseased plants

5.1 Introduction

Sexual seeds, which in scientific terms are simply called fertilised ovules, are a good source of plant reproduction. Unlike vegetative propagation materials, seeds have been known to be less risky in transmitting diseases to seedlings, although cassava seedlings derived from seeds are not as vigorous as plants from cuttings. However, some diseases are known to be spread through propagation by seeds in a number of crops. Seed disease transmission refers to passage of pathogens from seeds to seedlings and plants and this is usually done by infecting the seed embryo or infecting the seedling by mechanical injury (Hull, 2009). A potyvirus, *Soybean mosaic virus* infects all parts of a seed such as testa, embryo and cotyledon (Bashar, 2015).

Previous reports indicate that about one-seventh of known plant viruses are seed transmissible in at least one of their hosts (Hull, 2009). In the fields of Malawi, EACMMV, EACMCV, SACMV, CBSV and UCBSV are known to infect the cassava crop (Ogbe et al., 1997; Zhou et al., 1998; Mbanzibwa et al., 2009; Alabi et al., 2011; Aloyce et al., 2013; Mbewe et al., 2014). There is no evidence that the above mentioned viruses are transmitted from seeds to seedlings in cassava. However, a number of begomoviruses such as *Sweet potato leaf curl virus* (SPLCV) and *Tomato yellow leaf curl virus* (TYLCV) have recently been reported to be seed transmissible (Kim et al., 2015; Kil et al., 2016). Although at low rate, *Zucchini yellow mosaic virus* (ZYMV) in pumpkin is seed-transmissible (Tóbiás and Palkovics, 2003).

Many farmers use stem cuttings as source of planting materials. However, scientists, especially plant breeders, use botanical seeds in their breeding programmes in order to acquire genetic diversity. Using molecular techniques in some crops such as soybean and cowpea, makes it possible to detect the viruses in seedlings propagated from seeds from diseased plants in some varieties or samples but not in others (Sherf and Mcnab, 1986; Ojuederie et al., 2009; Salem et al., 2010). Therefore, the absence of species of viruses in seedlings of a crop may not indicate the total non-transmissibility through seeds. While many viruses are transmitted from seed to seedlings via the embryo, some viruses such as *Tomato mosaic virus* are transmitted mainly due to contamination of seedlings by

mechanical means (Hull, 2009). Mean seed-borne transmission rates of up to 15% and 80.77% have been reported for SPLCV and TYLCV in sweet potato and tomato respectively (Kim et al., 2015; Kil et al., 2016). Co-infection of seed-borne viruses occurs in some crops such as cowpea (Salem et al., 2010). Seed-transmission acts as the initial source of pathogen inoculum. Therefore, Understanding the transmission of viruses is good in disease resistance studies. Seeds of tolerant tomato varieties were reported by Kil et al. (2016) to have less amount of viral genome than susceptible varieties. This gives an indication that plant viruses accumulate less in seeds of resistant varieties. However, there is need to determine if these viruses are seed-transmissible in cassava. Knowledge of the seed-transmission will help in development of varieties that are tolerant/resistant to CMD and CBSD even by just studying the seeds. The aim of the study was to determine if SACMV, EACMCV, CBSV and UCBSV are seed transmissible.

5.2 Materials and methods

5.2.1 Planting materials and sampling

Cassava seeds from diseased plants were used for this experiment. Seeds from Mbundumali, MK06/0347, CH06/033 and Phoso genotypes were obtained from various cassava fields from plants showing CMD and CBSD foliar symptoms around Chitedze and Chitala Research Stations. The presence of SACMV and EACMMV was confirmed in the fields with plants from which seeds were obtained. A large number of seeds were obtained from each genotype in order to mitigate the effect of low germination rate. Therefore not every plant from which the seeds were obtained was virus tested. The choice of the genotypes depended on the ability to obtain enough seeds for the experiment.

Table 5.1 Description of the genotypes from which seeds were obtained

Variety	Tolerance/susceptibility
Mbundumali	Tolerant to CBSD but susceptible to CMD
MK06/0347	Susceptible to CMD
CH06/033	Susceptible to CBSD
Phoso	Tolerant to both CMD and CBSD

Haggblade and Zulu, 2003; Alene et al., 2013

Cassava seeds were sown in floating trays in a greenhouse, replicated three times in a completely randomized design (CRD) at Chitedze Research Station as indicated in Figure 5.1. After three weeks, 20 seedlings of each genotype were transplanted into polythene tubes. The plants were observed every week for foliar symptoms of CMD and CBSD. They

were allowed to grow for 90 days and thereafter the leaves were sampled and analysed for the presence of cassava mosaic geminiviruses and cassava brown streak viruses. Leaf samples for laboratory analysis were collected by taking a third fully expanded leaf from the top of each plant (Abarshi, 2012; Kumar et al., 2014).
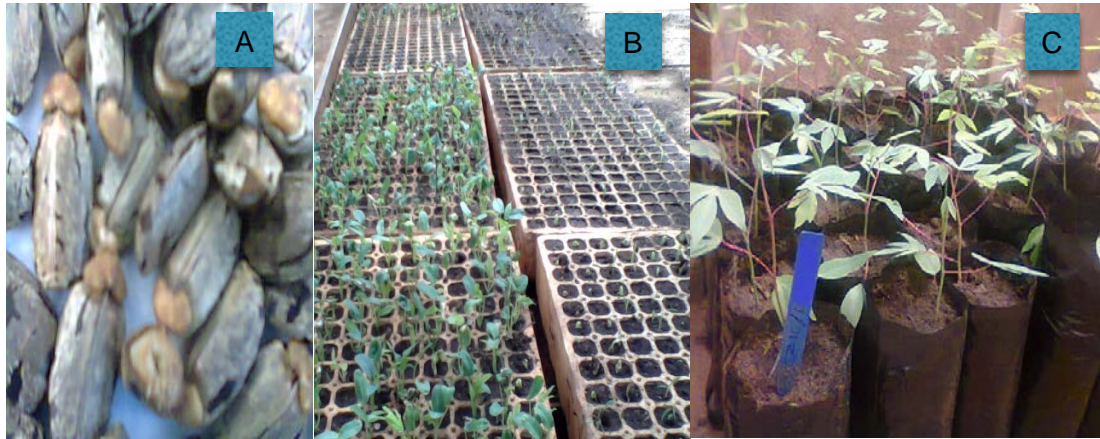
Figure 5.1 Cassava true seeds (A) cassava seedlings in floating trays (B) and cassava seedlings in polythene plastic tubes (C)

5.2.2 Total nucleic acid extraction and bulking

DNA and RNA were extracted from leaf samples from seedlings according to the procedure described in section 3.2.6. After nucleic acid extraction, the nucleic acids from five plant leaves on each replicate were pooled into four bulks to form a composite nucleic acid sample using equal volumes from each sample of 20 µl; making a composite sample of 100 µl. This means that each replicate of a cultivar had four composite samples.

5.2.3 Detection of *cassava brown streak virus* and *Ugandan cassava brown streak virus*

Detection of the CBSV and UCBSV in cassava seedlings was done using the TaqMan assay protocol using step one real-time PCR system as described in section 4.2.3.

5.2.4 Detection of *South African cassava mosaic virus* and *East African cassava mosaic Malawi virus*

Detection of the SACMV and EACMMV titre was done using SYBR Green I protocol using step one real-time PCR system as described in section 3.2.7.

5.3 Results and discussion

No foliar CMD and CBSD symptoms were observed in any of the plants. However, lack of disease symptoms does not necessarily mean a lack of transmission of the CMD and CBSD causative agents of interest from seeds to seedlings. This is because there is a possibility of a plant accommodating viruses which are not in large enough quantities to cause the symptoms (Halliwell and Gazaway, 1975). As a result, cassava seedlings were analysed for SACMV, EACMMV, CBSV and UCBSV but none of these viruses were detected in any of the four genotypes of cassava seedlings grown from seeds obtained from diseased plants. Figure 5.2 shows amplification of positive controls of CBSV and UCBSV only. Figure 5.3 shows amplification of the endogenous control, COX. Figure 5.4 shows amplification of positive controls of SACMV and EACMMV while Figure 5.5 shows amplification of the endogenous control, RubisCO. These results confirmed the findings by Maruthi et al. (2005) and Rwegasira (2009) who reported that that these viruses are seed transmissible. This might be due to the inability of the viruses to infect the seed through the embryo or other seed organs and to move into the seedling from the external surface of the seed through injury of the seedling during seedling development. Wang and Maule (1994) attributed the inability of viruses to be transmitted from parental plants to seeds, and subsequent transmission to offspring, to the action of maternal genes. This means that although there are many potyviruses and geminiviruses that are seed transmissible in a number of crops (Tóbiás and Palkovics, 2003; Bashar, 2015; Kim et al., 2015; Kil et al., 2016), these viruses are not able to do so in cassava.
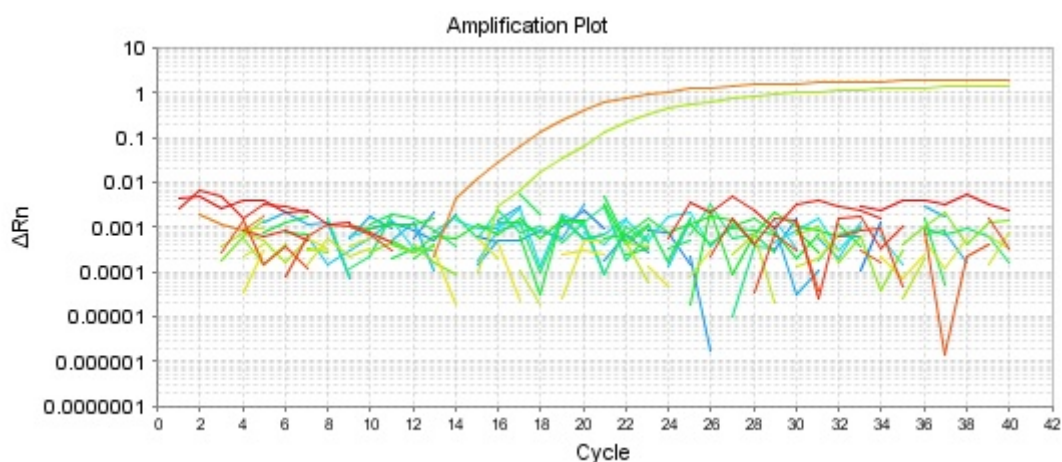

Figure 5.2 *Cassava brown streak virus* **and** *Ugandan cassava brown streak virus* **showing amplification of positive controls**
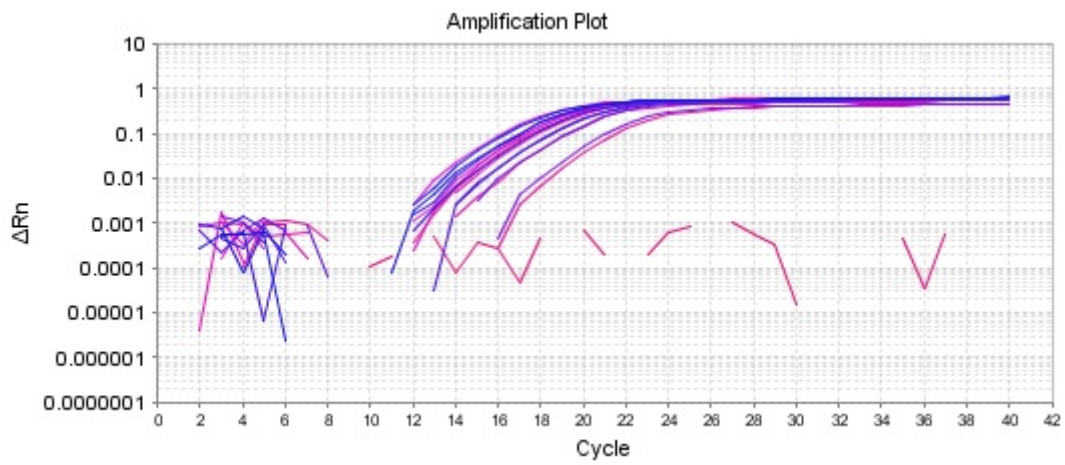
Figure 5.3 Amplification plot of the reference gene, cytochrome oxidase
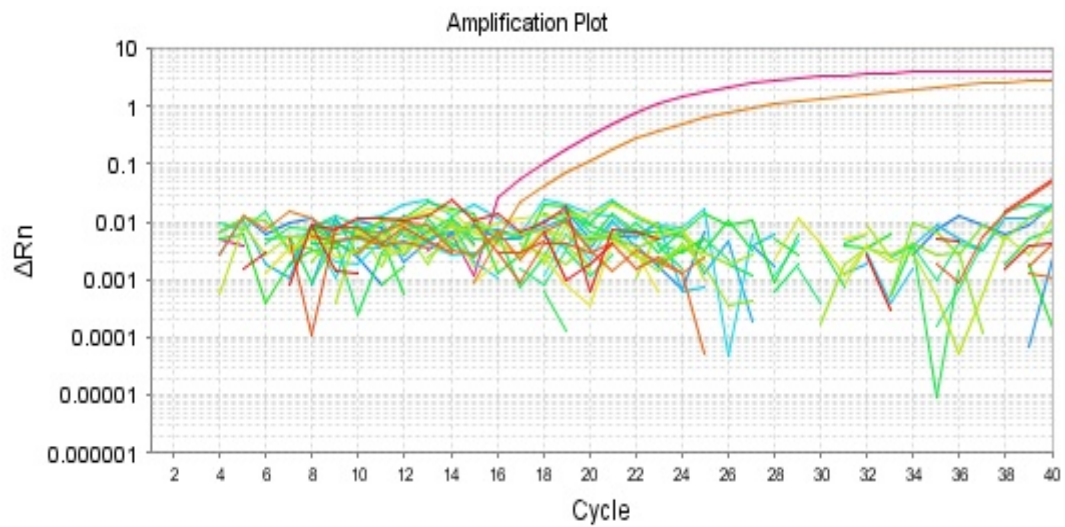
Figure 5.4 *East African cassava mosaic Malawi virus* **and** *South African cassava mosaic virus* **showing amplification of positive controls**
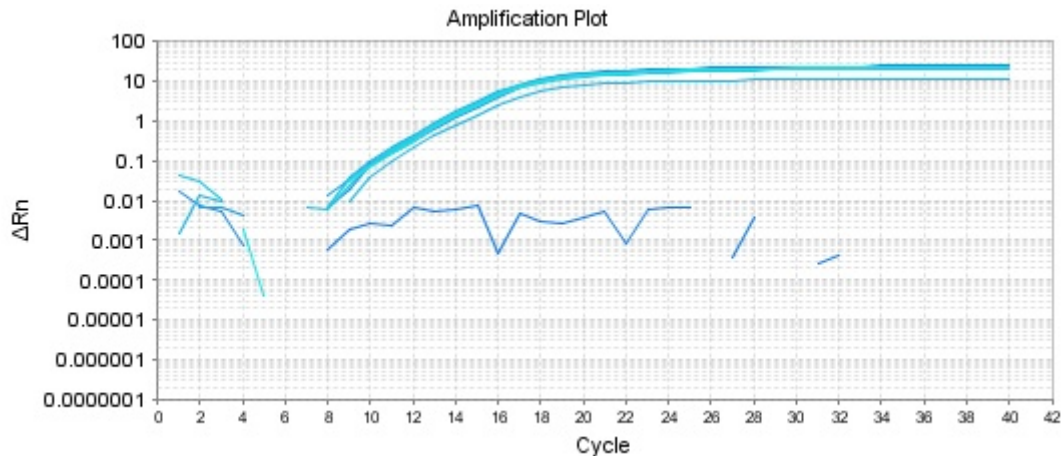
Figure 5.5 Amplification plot of the reference gene, Ribulose-1,5-bisphosphate carboxylase oxygenase

5.4 Conclusions and recommendations

Under the current experimental conditions in this study, SACMV, EACMMV, CBSV and UCBSV were not seed transmissible based on data from twenty plants per genotype used in this research. The four CMG and CBSV species prevailing in Malawi might not have the mechanism to move from either the infected plant to seeds or from seeds to seedlings. This gives some relief, as in cassava breeding true seeds play a vital role and are frequently used to create heterogeneity. If CMD and CBSD were confirmed to be seed transmissible, it would have posed a big challenge, as implementation of new strategies would have been necessary with regard to cassava breeding. However, further studies are recommended to look at the presence of the viruses in the embryo, seed coat and cotyledon.

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CHAPTER 6
General conclusions and recommendations

Cassava is an important crop in Malawi and other African countries. The production is, however, impeded by among other constraints, diseases such as CMD and CBSD. These two diseases are of significant concern in Malawi. In Malawi CBSV and UCBSV are known to cause CBSD while CMD is caused by EACMMV, EACMCV and SACMV. Efforts are underway to develop cassava varieties that are resistant or tolerant to both CBSD and CMD. There is no information on cassava released varieties or breeding parents on viral accumulation over a specific period of growth. No information on the quantities of viruses causing CBSD and CMD in various cassava tissues is available for varieties released in Malawi. Different organs of cassava are made up of different tissue cells. Knowledge of quantities of CBSV, UCBSV, EACMMV and SACMV in various tissues of cassava plants will assist in the study of resistance of genotypes to CMD and CBSD. Determination of variations in virus titre would also help in the diagnosis of CMD and CBSD without being restricted to only one plant organ. Cassava seeds are important, especially in plant breeding, due to their heterotic nature. They are used to propagate genetic variations created by crosses in a number of areas such as disease resistance, and are used for breeding activities.

Data on CMD incidence, severity and virus titre of F1 cassava progenies in the field were assimilated in this research. Disease incidence and severity were found to correlate. No symptoms of CBSD were observed and the two CBSV species were not detected at any stages of sampling. The progenies with high CMD severity symptoms had higher EACMMV and SACMV titres compared to those with low symptom severity. The graph constructed indicated that EACMMV and SACMV titre depends on the genotype used. The virus does not usually show a straight line increase, as at some point the titre decreased. Virus titre may decrease at some point during crop growth and development. This shows that virus quantities change over time depending on a number of environment factors such as temperature and moisture which affect virus multiplication in plants, apart from the internal activities of the host genotypes. Comparison of virus titre and disease symptom severity is an important factor in the development of crop varieties that are resistant or tolerant to particular virus species.

During the determination of virus titre in different cassava tissues, it was found that CBSV and UCBSV were positively correlated. The viruses were detected in all the tested tissues

but root parenchyma seemed to accommodate the highest CBSV and UCBSV quantity. The stem cortex of the upper part of the plant and leaves consistently produced high virus quantities just as the root parenchyma. EACMMV and SACMV were not evenly distributed and the virus quantities were high in the leaves especially the first fully open leaves. There is therefore the possibility of using any of the tissues tested in this experiment in CMD and CBSD diagnosis.

The analysis of seedlings grown from seeds taken from diseased plants showed that CBSV, UCBSV, EACMMV and SACMV were not seed transmissible. There were no symptoms of the four virus species in seedlings in the greenhouse. Unlike vegetative propagation, seed propagation is therefore safe and can help to control the transmission of the virus diseases.

Summary

Cassava (*Manihot esculanta* Crantz) is a perennial woody plant which is cultivated in Africa, Asia and Latin America. It is tolerant to drought and grows in poor soils. It is a source of carbohydrates, vitamins and small quantities of proteins. In Malawi, it is the most important root crop and it is grown across the country. It is consumed as a staple food by 30% of the people living along the central and northern part of Lake Malawi. Its production is constrained by among others, diseases CMD and CBSD. These two diseases are known to spread through white flies. The magnitude of attack by the two diseases depends on the genotype and the environment. No released genotypes in Malawi have ever been evaluated in seed transmission of viruses' studies. The experiment on the quantification of F1 cassava progenies from open pollinated F1 progeny crosses revealed that genotypes which show high severity of CMD are likely to allow rapid multiplication of EACMMV and SACMV. However, it has been proven that this might not always be the case as some genotypes may have higher virus quantities while showing slightly less severe symptoms. Genotypes which succumb to high disease symptom severity are likely to have high incidence of the disease. CMD symptom severity and incidence were positively correlated. The experiment on determination of virus quantities in different tissues has provided information that virus quantities in particular tissues depend on the cultivar used. The viruses can spread to all the tissues analysed in this research. It was shown that root parenchyma and leaf tissues accommodated most of CBSV and UCBSV in the cultivars used. When there was co-infection, CBSV and UCBSV were positively correlated. EACMMV and SACMV quantity can vary in cassava tissues depending on the cultivar. Leaves have shown to contain a relatively high quantity of the viruses. CBSV, UCBSV, EACMMV and SACMV symptoms were not observed in seedlings grown from seeds which were obtained from diseased plants. The virus species were also not detected using Real-time PCR. This suggests that these virus species are not transmitted from diseased plants through seeds.

Opsomming

Cassava (*Manihot esculanta* Crantz) is 'n meerjarige houtagtige plant wat verbou word in Afrika, Asië en Latyns Amerika. Dit is droogte tolerant en kan groei in swak grond. Dit is 'n bron van koolhidrate, vitamines en klein hoeveelhede proteïene. Dit is die belangrikste wortelgewas in Malawi, en dit groei oral in die land. Dit word as stapelvoedsel gebruik deur 30% van die mense wat woon langs die sentrale en noordelike dele van die Malawi meer. Cassava produksie word beperk deur, onder andere, die siektes CMD en CBSD. Hierdie twee siektes word versprei deur witvlieë. Die erns van die infeksie deur hierdie twee siektes hang af van die genotipe en die omgewing. Inligting oor die reaksie van verskillende Malawi genotipes en hulle nageslag aangaande akkumulasie van virusse, is baie beperk. Geen vrygestelde genotipes in Malawi is nog ooit geëvalueer vir saadoordrag van virusse nie. Die eksperiment oor die kwantifikasie van cassava F1 nageslag van oop en spesifieke kruisings het getoon dat genotipes met 'n hoë vlak van CMD 'n vinnige vermeerdering van SACMV toelaat. Dit is egter bewys dat dit nie altyd die geval is nie, omdat sekere genotipes groter hoeveelhede virus gehad het terwyl hulle steeds minder simptome getoon het; daar was dus geen korrelasie tussen min simptome van SACMV en CMD en virus insidensie nie. Genotipes wat baie siektesimptome gehad het egter hoë virus insidensie getoon; dus was die graad van die siekte met die insidensie gekorreleer. Die eksperiment vir die bepaling van virus hoeveelhede in verskillende weefseltipes het getoon dat virus hoeveelhede in spesifieke weefsel afhang van die cultivar wat gebruik is. Virusse het versprei na alle weefsel wat in die studie geanaliseer is. Wortel parenkiem weefsel het meeste van die CBSV en UCBSV in die cultivars gehuisves. Waar daar ko-infeksie was, was CBSV en UCBSV positief gekorreleer. SACMV hoeveelheid het gewissel tussen die wortel parenkiem, wortel korteks en stam korteks (5 cm van bo), afhangend van die cultivar. Blare het groot hoeveelhede virusse gehad. CBSV, UCBSV, EACMMV en SACMV simptome is nie gesien in saailinge wat van saad van siek plante ontwikkel is nie. Die virus spesies is ook nie met Real-time PCR gesien nie. Dit wys dat die virus spesies nie oorgdra is van siek plante deur saad nie.

APPENDICES

Appendix 1 Cycle threshold values for *East African cassava mosaic Malawi virus* in F1 cassava progeny crosses

Progeny	9 MAP	10 MAP	11 MAP	12 MAP
Mbundumali OP	20.69	15.98	20.00	18.70
Kachamba OP	23.69	18.90	20.08	21.45
Beatrice OP	29.06	25.96	27.16	30.55
Gushe OP	34.61	30.24	37.20	32.92
Chamandanda OP	32.70	27.78	34.78	34.16
MK06/0323 OP	37.00	27.10	29.30	37.97
Silira OP	35.88	27.20	34.70	34.39
Mbundumali (Control)	25.47	18.08	20.97	19.06
Mulola (Control)	29.93	27.12	28.35	28.00
Calibrator	29.06	28.12	30.09	30.88

Appendix 2 Cycle threshold values for *South African cassava mosaic virus* in F1 cassava progeny crosses

Progeny	9 MAP	10 MAP	11 MAP	12 MAP
Mbundumali OP	20.78	16.00	19.74	19.23
Kachamba OP	22.44	18.11	19.08	21.46
Beatrice OP	25.99	27.37	28.22	29.10
Gushe OP	34.00	29.79	28.81	37.46
Chamandanda OP	29.91	32.88	36.81	37.50
MK06/0323 OP	32.95	29.19	36.72	37.01
Silira OP	29.69	33.79	35.85	37.41
Mbundumali (Control)	25.92	18.97	20.41	19.45
Mulola (Control)	25.43	26.67	26.25	28.72
Calibrator	25.99	26.71	29.05	30.46

Appendix 3   Cycle threshold values for reference gene, ribulose-1,5-bisphosphate carboxylase oxygenase in F1 cassava progeny crosses

Progeny	9 MAP	10 MAP	11 MAP	12 MAP
Mbundumali OP	12.18	11.34	13.02	10.39
Kachamba OP	12.07	12.45	12.01	11.99
Beatrice OP	13.64	13.70	13.86	13.34
Gushe OP	13.01	13.66	13.00	12.45
Chamandanda OP	13.70	13.55	13.01	12.56
MK06/0323 OP	12.01	12.88	13.00	12.02
Silira OP	12.01	13.10	12.23	12.12
Mbundumali (Control)	13.50	12.77	13.30	11.01
Mulola (Control)	13.08	13.69	13.00	12.66
Calibrator	13.64	13.86	13.74	13.33

Appendix 4   Threshold cycle values for *Cassava brown streak virus*, *Ugandan cassava brown streak virus* and cytochrome oxidase in co-infected Mbundumali plant tissue

Tissue	CBSV	UCBSV	COX
1st fully open leaf	20.03	21.11	12.18
5th fully open leaf	19.83	21.21	12.35
10th fully open leaf	18.23	19.35	12.23
Stem bark (5 cm from top)	19.29	20.00	12.39
Stem bark (middle)	19.83	21.11	12.29
Stem bark (5 cm from roots)	19.95	21.44	12.49
Root parenchyma (Non-necrotic)	16.18	20.61	12.19
Root cortex (Peel)	20.04	22.57	12.51
Calibrator	20.57	21.06	12.04

Appendix 5 Threshold cycle values *Cassava brown streak virus, Ugandan cassava brown streak virus* and cytochrome oxidase in co-infected Mulola plant tissue

Tissue	CBSV	UCBSV	COX
1st fully open leaf	22.02	23.00	13.29
5th fully open leaf	22.16	22.74	13.47
10th fully open leaf	21.23	22.52	13.62
Stem bark (5 cm from top)	21.50	22.26	13.48
Stem bark (middle)	21.51	22.26	13.13
Stem bark (5 cm from roots)	22.14	23.62	13.53
Root parenchyma (Non-necrotic)	21.45	22.54	13.38
Root cortex (Peel)	23.45	24.19	13.72
Calibrator	22.16	22.74	13.47

Appendix 6 Threshold cycle values *Cassava brown streak virus* and cytochrome oxidase in single-infected Mbundumali plant tissue

Tissue	CBSV	UCBSV	COX
1st fully open leaf	24.25	-	13.84
5th fully open leaf	21.99	-	13.95
10th fully open leaf	21.60	-	13.54
Stem bark (5 cm from top)	21.91	-	13.67
Stem bark (middle)	22.84	-	14.02
Stem bark (5 cm from roots)	24.29	-	13.99
Root parenchyma (Non-necrotic)	22.26	-	14.17
Root cortex (Peel)	24.31	-	13.99
Calibrator	24.28	-	13.88

Appendix 7 Threshold cycle values for *Cassava brown streak virus*, *Ugandan cassava brown streak virus* and cytochrome oxidase in single-infected Mulola plant tissue

Tissue	CBSV	UCBSV	COX
1st fully open leaf	25.30	-	14.01
5th fully open leaf	25.19	-	14.19
10th fully open leaf	24.05	-	13.74
Stem bark (5 cm from top)	24.08	-	14.11
Stem bark (middle)	24.89	-	14.32
Stem bark (5 cm from roots)	25.06	-	13.88
Root parenchyma (Non-necrotic)	25.65	-	14.66
Root cortex (Peel)	25.37	-	14.32
Calibrator	24.97	-	13.97

Appendix 8 Threshold cycle values for *Cassava brown streak virus*, *Ugandan cassava brown streak virus* and cytochrome oxidase in single-infected Kalawe plant tissue

Tissue	CBSV	UCBSV	COX
1st fully open leaf	-	23.06	15.04
5th fully open leaf	-	21.90	14.79
10th fully open leaf	-	22.94	15.00
Stem bark (5 cm from top)	-	24.90	15.17
Stem bark (middle)	-	26.39	14.88
Stem bark (5 cm from roots)	-	27.30	14.77
Root parenchyma (Non-necrotic)	-	25.70	15.04
Root cortex (Peel)	-	27.55	14.94
Calibrator	-	23.93	14.59

Appendix 9 Threshold cycle values for *East African cassava mosaic virus*, *South African cassava mosaic virus* and ribulose-1,5-bisphosphate carboxylase oxygenase in co-infected Mbundumali plant tissue

Tissue	EACMMV	SACMV	RUBISCOL
1st fully open leaf	18.35	21.98	14.09
5th fully open leaf	23.94	25.27	14.33
10th fully open leaf	21.65	22.33	14.14
Stem bark (5 cm from top)	21.10	22.70	13.99
Stem bark (middle)	21.77	24.16	13.89
Stem bark (5 cm from roots)	23.73	29.02	14.55
Root parenchyma (Non-necrotic)	23.26	25.73	14.03
Root cortex (Peel)	25.17	26.01	14.24
Calibrator	25.46	26.13	14.24

Appendix 10 Threshold cycle values for *East African cassava mosaic virus*, *South African cassava mosaic virus* and ribulose-1,5-bisphosphate carboxylase oxygenase in co-infected Mulola plant tissue

Tissue	EACMMV	SACMV	RUBISCOL
1st fully open leaf	25.82	27.78	14.10
5th fully open leaf	24.63	25.07	13.67
10th fully open leaf	25.06	27.00	13.96
Stem bark (5 cm from top)	26.95	31.00	13.99
Stem bark (middle)	30.89	29.71	13.69
Stem bark (5 cm from roots)	34.49	31.40	13.88
Root parenchyma (Non-necrotic)	27.09	31.50	14.44
Root cortex (Peel)	29.10	31.58	14.07
Calibrator	24.63	25.07	13.67

Appendix 11 Threshold cycle values for *East African cassava mosaic virus, South African cassava mosaic virus* and ribulose-1,5-bisphosphate carboxylase oxygenase in co-infected Kalawe plant tissue

Tissue	EACMMV	SACMV	RUBISCOL
1st fully open leaf	18.02	20.03	14.18
5th fully open leaf	19.40	14.18	14.78
10th fully open leaf	20.54	22.28	14.55
Stem bark (5 cm from top)	21.12	22.83	15.03
Stem bark (middle)	21.27	22.63	15.19
Stem bark (5 cm from roots)	21.32	23.07	14.38
Root parenchyma (Non-necrotic)	22.49	24.42	15.33
Root cortex (Peel)	22.88	26.87	15.01
Calibrator	22.49	23.71	14.54