SCREENING OF FARMER-PREFERRED CASSAVA CULTIVARS FOR RESISTANCE TO CASSAVA BROWN STREAK VIRUS DISEASE IN KENYA

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DECLARATION

This thesis is my original work and has not been presented for a degree or any other award in any other University.

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DEDICATION

I dedicate this thesis to my mother Beth Mbiriri, my sister Lilian Waithera and to my grandfather Erastus Mbiriri.

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	Ascorbic acid
APX	Ascorbate peroxidase
bp	Base pairs
CAT	Catalase
CBSD	Cassava brown streak disease
CBSV	Cassava brown streak virus
cDNA	Complementary deoxyribonucleic acid
CMD	Cassava mosaic disease
CTAB	Cetyl trimethyl ammonium bromide
DHA	Dehydroascorbate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F	Forward
FAO	Food and Agriculture Organization
FW	Fresh weight
GoK	Government of Kenya
GPX	Glutathione peroxidase
GR	Glutathione reductase

GSH	Glutathione
H_2O_2	Hydrogen peroxide
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
MDA	Malondialdehyde
NaCl	Sodium chloride
PCR	Polymerase chain reaction
POD	Peroxidase
PVP	Polyvinylpyrrolidone
R	Reverse
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SOD	Superoxide dismutase
TAE	Tris-acetate- ethylenediaminetetraacetic acid
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
UCBSV	Uganda cassava brown streak virus
UV	Ultra- violet
wpi	Weeks post inoculation

ABSTRACT

Cassava brown streak disease (CBSD) is one of the seven major constraints threatening food security worldwide. The challenge in managing the disease is lack of an effective and sustainable method of mitigating CBSD with use of resistant cultivars being the most effective method. Despite decades of in depth studies on molecular characterization and diversity of cassava brown streak viruses (CBSV), there is limited information on physiological and biochemical changes in the CBSV- cassava pathosystem. The objective of this study was therefore to screen farmer preferred cassava cultivars for resistance to CBSV and further investigate the role of antioxidant enzymes in resistance of cassava to the virus. Thirteen cultivars of cassava were graft inoculated with CBSV infected cassava stems and screened for resistance to CBSD in the glass house. The disease symptoms were scored on a scale of 1 - 5 at 8 and 12 weeks post inoculation (wpi). The physiological parameters and antioxidant enzyme responses of the cassava cultivars following infection with CBSV were quantified at 8 and 12 wpi. From the study, cultivars Karibuni, Tajirika and Karembo were tolerant to CBSD, whereas cultivars TME 204, TME 7, TME 14, Kibandameno, KME-1, Ex-Mariakani, Ex-ndolo, MM96/7151, Ebwanatereka 1 and Ebwanatereka 2 were susceptible to CBSV. The amount of hydrogen peroxide (H_2O_2) was significantly higher ($P \leq 0.05$) in the inoculated susceptible (128.6-407.1nmol/g FW) than in the inoculated tolerant plants (231.7-275 nmol/g FW) at 8 wpi when compared to their respective controls. At 12 wpi, there was no significant difference ($P \geq 0.05$) in the amount of hydrogen peroxide in the inoculated plants of all the cultivars with respect to their controls. There was no significant difference ($P \geq 0.05$) in the amount of malondialdehyde in the inoculated (1.90-7.24 nmol/g FW) and non-inoculated plants (1.71-5.27 nmol/g FW) of all cultivars at 8 wpi. However, at 12 wpi, the amount of malondialdehyde was significantly higher ($P \leq 0.05$) in the inoculated susceptible plants (2.23-25.63 nmol/g FW) than in inoculated tolerant plants (3.36-5.71 nmol/g FW) when compared to their controls. The amount of chlorophyll was significantly lower ($P \leq 0.05$) in the inoculated (21.87-8.55 nmol/g FW) compared to the non-inoculated plants (24.06-12.28 nmol/g FW) of susceptible cultivars at 8 wpi and12 wpi. There was no significant difference ($P \geq 0.05$) in the amount of chlorophyll in the inoculated (10.29-10.84 nmol/g FW) and the non-inoculated (11.11-12.26 nmol/g FW) plants of tolerant cultivars. There was no significant difference ($P \geq 0.05$) in the amount of catalase in the inoculated (0.88-2.86 µmol/g FW) and non-inoculated (0.2-1.28 µmol/g FW) plants of all cultivars at 8 wpi. However, at 12

wpi the amount of catalase was significantly higher ($P \leq 0.05$) in the inoculated (1.61-3.44 µmol/g FW) compared to the non-inoculated (0.39-1.38 µmol/g FW) plants of all cultivars. The amount of peroxidase and was significantly ($P \leq 0.05$) higher (2.42-2.67 µmol/g FW) and (2.17-2.42 µmol/g FW) in inoculated plants of tolerant cultivars at 8 and 12 wpi respectively. The amount of ascorbate peroxidase was significantly ($P \leq 0.05$) higher (4.86-6.85 µmol/g FW) and (6.75-7.64 µmol/g FW) in inoculated tolerant plants at 8 and 12 wpi respectively compared to the non-inoculated tolerant plants. There was no significant difference ($P \leq 0.05$) in the amount of peroxidase and ascorbate peroxidase (1.34-2.74 µmol/g FW) and (2.18-5.26 µmol/g FW) respectively at 8 wpi and (1.17-2.52 µmol/g FW) and (2.68-6.85 µmol/g FW) respectively at 12 wpi in inoculated compared to the non-inoculated plants of susceptible cultivars. Karibuni, Tajirika and Karembo were identified as tolerant cultivars and had an increase in the amount of peroxidase and ascorbate peroxidase enzymes upon inoculation with CBSV. Cultivars tolerant to CBSD showed lower lipid peroxidation compared to the susceptible ones. The accumulation of hydrogen peroxide was lower in the infected tolerant plants compared to the susceptible plants. The amount of chlorophyll in susceptible plants reduced dramatically upon CBSV infection, whereas there was little or no reduction in chlorophyll in their tolerant counterparts. Therefore, ascorbate peroxidase and peroxidase enzymes could play a vital role in tolerance of cassava plants to CBSV infection. The two enzymes therefore could be useful tools in breeding strategies by plant breeders to induce resistance to CBSD in cassava cultivars susceptible to the disease. The two enzymes are also useful in agriculture for selection of cassava cultivars resistant to CBSD.

CHAPTER ONE: INTRODUCTION

1.1 Background information

Cassava (*Manihot esculenta* Crantz) is a hardy herbaceous plant majorly cultivated due to its high starch level (Lukuyu *et al*., 2014). The crop originated from Brazil in the 16th century and was brought to Africa together with maize (Akoroda and Ikpi, 1992). Cassava ranks second after maize as a food security crop in Africa (Howeler, 2001). In Africa, cassava is a significant food security crop due its potential to grow in marginal areas and to tolerate dry weather conditions (Hillocks, 2000). The crop is largely grown by resource-constrained farmers in tropical regions of the world including Kenya (Mdenye *et al*., 2016). Cassava can be grown all year round and therefore can serve as an alternative food when other foods are not available due to climate changes. Globally, cassava is cultivated on approximately 19 million hectares of land (Zhu *et al*., 2015). West Africa, South America and South East Asia are the major producers of cassava in the world with Africa producing more than half of the world's cassava (Guira *et al*., 2017).

The crop is susceptible to over 20 viral diseases with cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) having a disastrous effect on cassava production in Kenya and other cassava growing countries in sub-Saharan Africa (Hillocks and Thresh, 2000). As reported by Patil *et al.* (2015), the two viruses can cause global economic losses of roughly US$1 billion annually. Cassava brown streak disease (CBSD) is the most economically important diseases of cassava in the East African coast (Winter *et al*., 2010). The causal agent of the disease is Uganda cassava brown streak virus (UCBSV) and cassava brown streak virus (CBSV).

Cassava brown streak disease had received much less attention than cassava mosaic disease since it was earlier restricted to the East African lowlands (Patil *et al*., 2015). The virus however spread rapidly to East and Central Africa since 2004 posing a risk to the food security of many farmers. In many CBSD affected countries including Kenya, the spread of the disease has been controlled by sensitizing farmers about the disease, rouging, use of clean planting materials and implementing strict quarantine (Hillocks *et al*., 2002). In Kenya, CBSD affects many improved CMD resistant and highly adopted cultivars like TME 204 and TME 14 (Ntawuruhunga and Legg, 2007) and thus pose a great threat to food security. The rapid spread of the disease in Kenya requires that efforts be made to mitigate its further spread and destruction to cassava. To attain this goal, reliable sources of tolerance to cassava brown streak disease (CBSD) should be sought in both local and introduced cassava germplasm, and when identified, should be used as breeding stocks in the improvement of farmer-preferred cassava cultivars in Kenya.

Selection of resistant cassava cultivars could provide a simple, cost-effective and environmental-friendly strategy in controlling cassava brown streak disease (Kaweesi *et al*., 2014). Insight into the physiological and biochemical alterations among cassava cultivars upon challenge with CBSV virus can be used as markers of viral resistance or tolerance which will be of use in the selection of genetic material for dissemination to farmers or in breeding programs.

This study was carried out to determine resistance of farmer-preferred cassava to CBSD in Kenya and further establish the quantitative estimations of physiological and biochemical parameters such as chlorophyll pigments, lipid peroxidation and hydrogen peroxide and antioxidant enzymes, indicating their role in CBSV inoculated and non-inoculated plants of susceptible and resistant cultivars in order to determine biochemical markers for resistance. Such

findings could contribute to improving the breeding strategies for farmers in order to curb the cassava brown streak disease menace. The mechanisms involving the defense responses in cassava cultivars with resistance to CBSD are not well understood.

1.2 Problem statement

The human population is rapidly increasing creating more demand for food. Cassava has been identified as a crop that can help solve food insecurity in Africa since it can be grown and harvested all year round and requires minimal inputs to cultivate. The crop can also grow in marginal land in cases where arable land is intruded by humans.

Over the years however, cassava plant has been threatened by several viral diseases that limit the crop yield. Cassava brown streak disease is one of the major diseases facing cassava. It has been reported to be among the seven major threats to food security in the world. It leads to root yield losses of approximately 90% which renders roots unfit for human consumption and for use as animal feed (Irungu, 2011). The disease causes economic losses of up to US$75 million in East and Central Africa. However, until recently CBSD had not been given much attention like other cassava viral diseases such as cassava mosaic disease (Hillocks and Jennings, 2003). For example, between 1990 and 2003, twelve high yielding and CMD resistant cultivars were released but were unfortunately susceptible to CBSD, as they were not selected for CBSD resistance.

1.3 Justification

Cassava is a salient source of carbohydrates in developing countries mostly to the resource constrained subsistence farmers. Cassava is ranked as the third richest source of carbohydrates in Africa after maize and rice. In sub-Saharan Africa, cassava has been identified as a major food security crop due to its adaptability to marginal areas. As a result, it has been widely grown in tropical and sub-tropical areas. However, the role of cassava in food security more so to the poor is under threat due to an increase in both biotic and abiotic constraints that greatly limit attainment of optimal yields among them the CBSD and UCBSD. Among the strategies to control CBSD, breeding for resistance offers an effective and sustainable management strategy (Devries and Toenniesen, 2001). However, before a breeding programme is initiated, there is need to screen both local and introduced germplasm to identify possible sources of resistance or tolerance. Identification of sources of resistance to CBSD will help in eradication of the disease leading to an increase in cassava production and reducing famine during droughts. This will increase economic output and improve livelihoods of poor farmers.

Plants have over the years developed chemical and physical barriers that include inducible defense responses that impede pathogen colonization (Vanitha *et al.,* 2009). This however requires all inclusive studies and insight into the adaptive mechanisms and responses of these plants to pathogen invasion. Currently, there is no information available regarding physiological and biochemical alterations in cassava plants infected by CBSV while information on cassava brown streak resistance status of local cultivars is also inadequate.

1.4 Objectives

1.4.1 Broad objective

The broad objective of this study was to evaluate the resistance of farmer preferred cassava cultivars to cassava brown streak virus in Kenya and explore the use of antioxidant metabolism as a way of identifying sources of resistance to cassava brown streak disease.

1.4.2 Specific objectives

i. To evaluate the response of farmer-preferred cassava cultivars to cassava brown streak virus inoculation.
ii. To determine the antioxidant response of cassava cultivars following infection with cassava brown streak virus.

1.5 Research questions

i. Are farmer - preferred cassava cultivars resistant to cassava brown streak disease?
ii. Do plant antioxidant enzymes play any role in resistance of cassava to cassava brown streak disease?

CHAPTER TWO: LITERATURE REVIEW

2.1 Economic importance of cassava

Cassava ranks second in the world as the most significant root crop after potatoes and second most important staple crop in Africa after maize (*Zea mays*) (Kibet *et al*., 2017). In the world, cassava provides nourishment to over 800 million people; with 40 % of the African population depending on cassava as a food crop (Burns *et al*., 2010). Its increasing popularity can partly be attributed to its ability to grow on marginal land with limited resource input that characterizes many smallholder farmers (Devries and Toenniesen, 2001; Nassar and Ortiz, 2007). Therefore, cassava plays a major role in food security especially in areas prone to famine, drought and in times of civil disturbances.

Cassava root contains 30-40% dry matter weight containing 25.7-30% starch (Fakir *et al*., 2012). It is a major and the cheapest source of starch to a human population of over 800 million worldwide contributing over 500 kcal per day (FAO, 2009). In addition, cassava leaves are consumed as vegetables in many areas because they contain high amounts of vitamins (A and C), minerals (iron, zinc, calcium, potassium) and proteins (Montagnac *et al*., 2009). The crop can be used for food as: cooked fresh roots, cooked fresh leaves as vegetables, medicine, drinks (with cassava components), fermented pastes, unfermented and fermented cassava flour which is the most widely used product, sedimented starches and as granulated cooked or roasted cassava (Hillocks *et al*., 2002).

2.2 Cassava production in Kenya

Cassava is a key food crop and an income-generating crop for smallholder farmers in Kenya. In Kenya, it ranks third as a major food security crop after maize and Irish potatoes (GoK Report, 2010). It is grown on about 637259 ha of land with an output of 858461 tons annually (FAO, 2014). It is cultivated mainly in Western and Coastal regions of Kenya for commercial purposes, whereas in other regions it is produced in small quantities alongside other staples (Mulu-Mutuku *et al*., 2013). Cassava is grown majorly in the semi-arid areas because it is tolerant to drought and thus serves as a food security crop. Its production in Kenya is below the potential yield due to poor yield potential of popular cultivars, susceptibility to pests and diseases particularly mosaic disease (CMD) and brown streak disease (CBSD) and poor crop management practices among other constraints (Mware *et al*., 2009a).

2.3 Abiotic and biotic constraints to cassava production in Kenya

Some of the abiotic constraints limiting cassava farming in Kenya include drought, poor soil fertility due to overexploitation of marginal soils resources without replenishing them; deterioration of roots during storage; lack of disease-free materials and improved cultivars for planting and poor farming practices. Some of the poor farming practices adopted by farmers include improper spacing; improper weeding; and improper application of fertilizers; poor land preparation and improper processing of harvested roots (Mkamilo and Jeremiah, 2005). Post-harvest physiological deterioration is a challenge to farmers since it occurs naturally resulting in blackening of the harvested roots due to oxidation of phenolics (Bull *et al*., 2011). Cassava roots have a life span of 72 hours after harvesting and therefore needs to be further processed for a longer shelf life. Improper cropping systems and a missing value added chain that connects

farmers to local and international markets also poses a threat to cassava production in Kenya (Mwango'mbe *et al*., 2013).

The biotic constraints affecting cassava production in Kenya include the green cassava mite and cassava mealy bugs which are the major pests of cassava (Legg and Thresh, 2003). Cassava bacterial blight and cassava viral diseases are the major cassava disease constraints limiting cassava yields. In Africa, CBSD has been recorded as the most important viral disease facing cassava and a major contributor to food insecurity (Patil *et al*., 2015). Limited access to clean planting materials by farmers poses a challenge in production of cassava in Kenya. Kenya Agricultural and Livestock Research organization (KALRO) which is the main supplier of cassava cuttings lacks enough planting materials to meet the high demand in Western and Coastal regions. Cassava brown streak virus and whiteflies are the main threats to obtaining clean planting materials in Kenya (Mulu-Mutuku *et al*., 2013; Lagat *et al*., 2017).

2.4 Cassava pests and diseases

The major pests affecting cassava are cassava mealy bugs and cassava green mite. Cassava mealy bug (*Phenacoccus manihoti*) originated from South America and spread to sub-saharan Africa in the 1970s. It affects cassava production by distorting shoots, causing chlorosis and curling of leaves and stunting and weakening of stems. It causes yield losses of up to 80% in the absence of its natural enemies. There is no known cassava cultivar that is resistant to the mealy bugs (Parsa and Winotai, 2012). Cassava green mite (*Mononychellus tanajoa*) alone can cause yield losses of 45%. It was also introduced to Africa in 1970. The mites feed on young leaves and green stems of cassava. They reduce root yields by 80% and damage the leaf surface thus reducing the rate of photosynthesis (Onzo and Sabelis, 2005).

Cassava bacterial blight, cassava mosaic disease and cassava brown streak are the major diseases threatening cassava production. Cassava bacterial blight is caused by the bacteria *Xanthomonas axonopodis.* The disease causes wilting and leaf fall of cassava plants. It also causes reduction in root yield thus causing economic losses and availability of cuttings for the next planting season (Fanou *et al*., 2017). Cassava mosaic disease is caused by cassava mosaic virus. The disease is characterized by leaf chlorosis accompanied by leaf curling and stunting. The disease causes yield losses and reduces the plant vigour (Thresh and Cooter, 2005)

2.5 Cassava brown streak disease

The disease is caused by dual or single infection of the plant by *Uganda cassava brown streak virus* and *Cassava brown streak virus* as (Patil *et al*., (2015). The two viruses are single stranded positive sense RNA viruses. They are monopartite viruses enclosed in a capsule inside flexuous rod particles. They belong to the Potyviridae family and are in the genus *Ipomovirus* (Abarshi *et al*., 2012). Most CBSV and UCBSV exhibit 30% variability with each other at the nucleotide level but differ majorly in the P1 and Ham1h gene sequences. Uganda cassava brown streak virus isolates show up to 86 - 99% similarity at the coat protein gene level. The UCBSV isolates are ubiquitous in East Africa. As reported by Yadav *et al*. (2011), CBSV are mostly endemic to the coast of Tanzania and Mozambique.

Cassava brown streak disease is a major cause of food insecurity in the world (Yadav *et al*., 2011; Ogwok, 2015) and had only been recorded in Uganda, Kenya, Mozambique and Tanzania (Monger *et al*., 2001). In the recent past, CBSD has been recorded in Burundi, Democratic Republic of Congo, South Sudan, Rwanda and Mayotte Island (Tomlinson *et al*., 2018). Nucleic acid assays such as RT-PCR are used to detect the viruses. Reverse Transcription-PCR assays

have been developed to detect CBSV and UCBSV. Primers that are specific to each species of CBSV are available making simultaneous detection of both viruses in a sample possible through a two-step RT-PCR. To detect and quantify both CBSV and UCBSV even in very minute quantities, a real time RT-PCR was developed recently. This method has successfully detected CBSV in asymptomatic CBSV- infected leaves and also in newly emerging leaves (Irungu, 2011; Abarshi *et al*., 2012).

2.5.1 Epidemiology and transmission of cassava brown streak viruses

Cassava brown streak disease remained endemic in East Africa being majorly limited to the coastal lowlands for a long time. There are several reports of CBSD emergence and re-emergence in areas it had not been reported or had been declared endemic (Munganyinka *et al*., 2017). Mware *et al*. (2009b) reported a 46-100% CBSD incidence in Western Kenya but samples from Eastern and Central regions in Kenya were negative for CBSV. The report showed rapid spread of the virus from the coastal lowlands to regions thought to be endemic to CBSD. The reason for the recent and rapid spread of CBSD over a wider geographical region is poorly understood.

Use of susceptible cultivars, increase in disease pressure and increase in number of whiteflies have been suggested as the leading factors for the high incidence of CBSD (Katono *et al*., 2015). Transfer of the virus by planting cassava from stem cuttings is the major method of spread of CBSD. Cassava brown streak virus can be transmitted mechanically using farm tools like knives if they are used to cut infected stems then used to cut healthy plants (Maruthi *et al*., 2014). Whiteflies (*Bemisia tabaci*) can also spread the virus from an infected plant to a healthy one. During cross-breeding of different cultivars of cassava, the virus can be spread if the plant used

for grafting is infected. The virus can also be spread from multiplication centers in case the initial source of cassava was infected or was not checked for CBSV infection before dissemination to farmers.

2.5.2 Symptoms of cassava brown streak disease

The disease got its name from the brown lesions that are evident on the young stem which was the first disease symptom to be observed on cassava infected by the virus. On the contrary, these brown streak symptoms are not the major disease symptoms of CBSD. The major symptoms of CBSD usually appear on the older lower leaves but not the younger emerging leaves. This makes diagnosis of the disease difficult especially if the older symptomatic leaves are shed (Hillocks *et al*., 1996). Symptoms exhibited by cassava leaves infected with CBSD include feather-like chlorosis on the veins, or circular blotches of chlorosis between the primary veins (Figure 1). Stems display brown streaks of necrosis or die-back in extreme situations. In the root tubers, symptoms include brown to dark brown necrotic lesions (Figure 1 A), constrictions and reduction in starch and cyanide (Yadav *et al*., 2011; Patil *et al*., 2015).

The disease reduces the vigour of the plant cuttings for the next planting season. These symptoms however are different based on the type of cassava, strain of the virus isolate, age of the plant and the environmental parameters (Patil *et al*., 2015). The symptoms are more pronounced in the rainy season especially in high altitude areas but the plant recovers during the dry season. During the dry season, the plants shed the leaves and the new leaves exhibit no disease symptoms making diagnosis difficult. In susceptible cultivars, symptoms may be expressed in all parts of the plant, whereas in tolerant/resistant cultivars, the symptoms may be limited to the leaves.

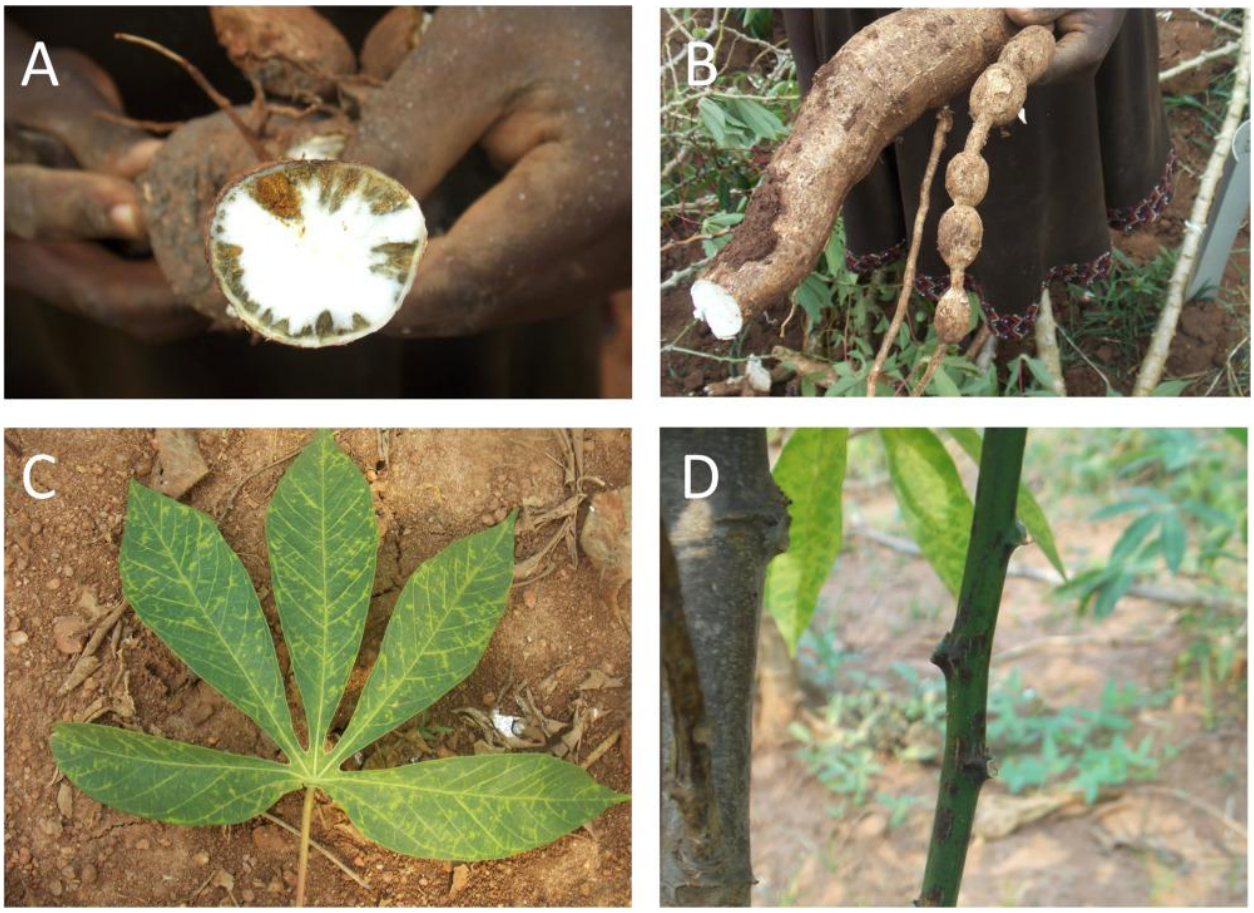

Figure 1: Cassava brown streak disease symptoms.

(A) Storage root necrosis, (B) radial root constrictions, (C) foliar chlorosis and (D) brown streaks or lesions on stems.

Source: Tomlinson *et al*. (2018).

2.5.3 Economic importance of cassava brown streak disease

Cassava brown streak disease suppresses yields by 30% - 85% (Yadav *et al.*, 2011). It is among the seven major causes of food insecurity in the world (Yadav *et al*., 2011; Ogwok, 2015). The disease damages roots, leaves and stems of cassava plant resulting in necrosis and constriction reducing the quality and total yield of the crop. The diseased roots are unfit for human and animal consumption thus cannot be sold by farmers (Wasswa *et al*., 2010). Losses caused by

necrosis of the roots become evident after harvesting the crop (Irungu, 2011). This is a great loss to the resource poor farmer due to the time and resources spent in cultivating the crop until it is mature. The losses are mostly severe for local cultivars whereby necrosis of the leaves increases from six months after propagation forcing the farmer to harvest the crop pre-maturely.

2.5.4 Management of cassava brown streak disease

Several management strategies have been employed to control CBSD with varying degrees of effectiveness. Utilization of clean planting materials is one of the best methods of controlling CBSV whereby clean cultivars are used for propagating new plants or for cross-breeding by grafting. The CBSV has also been managed by rouging infected plants to lower the possibility of the virus being spread from the infected plants to other healthy plants in the field. Practicing hygiene in the farm is a strategy that has been used by the farmers whereby farming tools are sterilized over fire to kill viruses before the tools are used to cut healthy plants.

Quarantine measures have been put in place especially in the borders whereby there is restriction in the transport of cassava plants from CBSD infested regions to neighboring regions (Tadeo, 2014). Thermotherapy has recently been reported to be an effective method of eradicating CBSV whereby infected plants are subjected to hot-air treatment at 36˚ C for 8 hours (Okori and Nakabonge, 2016). Yadav *et al*. (2011) also showed that RNA interference technique could be an effective method for controlling UCBSV. However, of all strategies put in place, usage of resistant cultivars is the most feasible and efficient method of mitigating CBSV.

2.6 Plant - virus interaction

Plant viruses evolve rapidly; probably due to their short replication cycles, wide host range and also due to presence of many of their particles in a single cell. Therefore, plant viruses produce new avirulent factors thus breaking the host resistance mechanism (Stange, 2006). Some plants have developed resistance genes which bind to the avirulent gene of the virus thus resisting viral infection or enhance gene silencing as a defense mechanism against evolving viruses. Over the years, plants have developed both physical and chemical barriers as mechanisms to defend themselves against viral infections. In incompatible interactions, the plant uses the hypersensitive response that involves localization of the infected cells and their death to prevent further spread of the virus (Stange, 2006).

Viruses are able to overcome very complex defense mechanisms by the host. RNAi silencing has been one of the strategies used by the host to overcome viral infection. The mechanism involves breaking down the RNA duplex formed during replication of positive stranded viral RNA. The plant in defense produces a complex of RNA helicases and RNA dependent RNA polymerase that degrades the viral RNA into small fragments (RNAi) (Hammond *et al.*, 2000; Pallas and Garcia, 2011). This mechanism has been used to date to control plant viral infections.

Upon infection and integration of the virus in a plant, the plant may fail to recognize the virus which is an advantage to the virus; this results into a compatible interaction. However, if the virus is recognized by the plant, an incompatible interaction ensues which is unfavorable to the virus. Susceptibility or resistance of a plant to viruses depends primarily on the host genotype. The plant may either produce active or passive defense against a virus. Passive defense occurs when the plant fails to produce factors needed for reproduction and spread of the virus in the

host. Active defense occurs when the plant recognizes the virus and produces defense genes that destroy the virus (Gergerich and Dolja, 2006; Paudel and Sanfacon, 2018).

Symptoms of viral infected plants range from being asymptomatic, necrotic lesions at the site of infection and may spread systemically in the whole plants. Typical viral infection symptoms in plants foliage include yellowing, mosaic patterns, streaks or stripes vein banding, vein clearing, leaf curling and rolling and chlorotic/necrotic lesions. Stems may exhibit grooving, pitting and tumors. Vegetables and fruits may exhibit discoloration, stunting, malformations and chlorotic ring spots. The result is reduced crop quality and yields and subsequent economic losses to the farmers (Gergerich and Dolja, 2006).

2.7 Role of reactive oxygen species and antioxidant enzyme in tolerance and resistance of plants to viruses

Plants subjected to biotic stresses such as virus infections can have excessive production of reactive oxygen species (ROS), which leads to oxidative stress (Goldbach *et al*., 2003). Production of reactive oxygen species occurs when plants are attacked by pathogens (Bolwell *et al.*, 2002; Torres *et al*., 2006). Nevertheless, the precise involvement of ROS in symptomatology and pathological process, particularly in susceptible plant–virus interactions is not well understood. Reactive oxygen species are potentially harmful to the cell, as they can hinder the usual cell functioning by oxidizing proteins, carbohydrates, lipids as well as nucleic acids, eventually leading to cell death (Amoako *et al*., 2015).

In susceptible virus-plant host associations, oxidative stress is expressed as an increase in reactive oxygen species which leads to lipid peroxidation, increased levels of hydrogen peroxide

and an imbalance in antioxidant enzymes (Neill *et al*., 2002; Hernandez *et al*., 2006). An increase in ROS has also been suggested to be caused by disturbances in the photosynthetic system during viral pathogenesis (Torres *et al*., 2006). Chlorosis and mosaic symptoms in plants are often associated with viral infection and are thought to be caused by an increase in ROS. In a controlled way, however, ROS play a role in physiological responses and early signaling pathways in response to pathogen infections thus can trigger systemic acquired resistance (Sandermann, 2000). In plants resistant to viruses, ROS might play an important role in resistance (Riedle-Bauer, 2000).

Hydrogen peroxide produced during plant viral infection may either lead to signaling leading to localized cell death or induce defense in adjacent cells (Levine *et al*., 1994). Therefore, tight control rather than complete removal of ROS is crucial in preventing oxidative damage in plants and at the same time allowing ROS to execute its key role as a signal molecule (Mittler *et al*., 2004). In order to maintain a proper balance between ROS generation and mitigation as well as repair of the oxidative damage initiated by ROS, plants possess a set of antioxidant and scavenging enzymes which include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) (Asada, 2006; Sofo *et al*., 2015). Report by Radwan *et al*. (2010) suggests a significant role of antioxidant and scavenging enzymes in plant resistance to pathogenesis.

Regulation of antioxidant enzymes could play a role in compatible and incompatible plant-virus interactions. Elevation of antioxidant enzymes during plant-virus interaction is thought to increase resistance of the plant to the pathogen (Barna *et al*., 2003). A decrease in antioxidant enzymes followed by an increase in ROS might contribute to viral infection (Neill *et al*., 2002).

De Gara *et al.* (2003) suggested that changes in the activities of ROS-detoxifying enzymes might be necessary in eliciting phytopathogenic response. Peroxidases in particular have been implicated in formation of ROS. Upon oxidation of phenolic compounds, peroxidase decomposes hydrogen peroxide. Ascorbate peroxidase has a higher predisposition towards hydrogen peroxide reducing it to water in mitochondria, chloroplast and cytosol using ascorbate as the specific electron donor (Sofo *et al*., 2015). The balance of CAT, APX and GPX activities, which represent hydrogen peroxide scavenging enzymes, is important for burking toxic hydrogen peroxide amount in a cell.

These enzymes however scavenge hydrogen peroxide using different mechanisms. Ascorbate peroxidase requires ascorbate glutathione cycle; GPX detoxifies H_2O_2 to water and oxygen but using GSH as the reducing agent. Catalase converts H_2O_2 directly into water and oxygen, whereas APX is more of a detoxifying enzyme than a signaling molecule. Hydrogen peroxide is a signaling molecule in both abiotic and biotic stress that leads to various responses in a cell depending on where it is produced (Sofo *et al*., 2015).

2.8 Effect of viral infection on chlorophyll content

The yellowing and chlorosis observed during viral infection is as a result of interference with photosynthetic activity. The most common symptom of plant viral infection is chlorosis (Zhao *et al*., 2016). Plant viruses affect photosynthesis by reducing the starch content, the synthesis of chlorophyll and slowing down photosynthesis. The reductions in chlorophyll due to CBSV infection is mainly as a result of damage to chloroplasts leading to reduced functioning of the chloroplast. Malfunctioning of chloroplasts is debilitated by decrease in the number of chloroplasts, distortion and loosening of thylakoids and disappearance of stroma, complete

distortation of chloroplast shape and reduced chloroplast size accompanied by scattering of grana in the cytoplasm (Sofy *et al*., 2014).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Evaluation of the response of Kenyan farmer-preferred cassava cultivars to cassava brown streak virus inoculation

3.1.1 Cassava germplasm

Thirteen cassava cultivars comprising of local landraces and elite genotypes from Kenya Agricultural and Livestock Research Organization (KALRO), Biotechnology Centre were established in the glasshouse at the University of Nairobi, Chiromo campus. The cultivars (Ebwanatereka 1, Ebwanatereka 2, Kibandameno, TME 14, TME 7, TME 204, Ex-Mariakani, Ex-ndolo, MM96/7151 and KME-1 Karibuni, Karembo and Tajirika) were selected based on some traits that are preferred by farmers (Table 1). The cultivars are high yielding, they have high starch levels, good marketability, and the roots have desirable taste, good cooking quality, mealiness and good texture (Marigi *et al*., 2016; Nyaboga *et al*., 2013). The stems were cut into smaller nodal sections using a machete. The cuttings comprised of two upper nodes for shoot formation and two bottom nodes for root formation. The stem cuttings were planted in plastic pots (20.5 cm wide) containing sterilized forest soil mixed with sterilized farmyard manure in the ratio of 1:3 (v/v). The planted stem cuttings were covered with plastic bags to maintain high humidity necessary for faster sprouting and constantly watered three times in a week. After two weeks of sprouting, the plastic bags were removed to allow growth and expansion of the shoot. The plants were maintained in the glasshouse for six weeks before inoculation (Figure 2).

Table 1: Origin, farmer-preferred traits and reaction to diseases of selected cultivars in Kenya

Cassava cultivar	Type	Traits and reaction to diseases
Karibuni	Landrace	High tuber yield, matures in 8-12 months, sweet, high branching and excellent for inter-cropping; tolerant to CMD, CBSD status unknown
Tajirika	Landrace	High yields, matures in 8 months and has straight stems ideal for inter-cropping; CMD tolerant, CBSD status unknown
Kibandameno	Landrace	High yields, low cyanide content, high dry matter, sweet, matures in 8 months. high bulk, and good marketability; CMD susceptible, CBSD status unknown
TME 14	Elite	Sweet, high yielding, white cortex, low cyanogen, high dry matter and matures in 9 months; CMD tolerant, CBSD status unknown
TME 7	Elite	High yields; CMD resistant, CBSD status unknown
TME 204	Elite	High yield, early maturing, sweet, soft texture and good quality flour; CMD resistant, CBSD status unknown
Karembo	Landrace	Matures in 8 months, high tuber yield and sweet; CMD tolerant, CBSD status unknown
Ebwanatereka	Landrace	Mealiness, high yielding, high dry matter and sweet; CMD susceptible, CBSD status unknown
KME-1	Elite	Sweet, less fibrous and low cyanide content' CMD susceptible, CBSD status unknown
MM96/7151	Elite	High yields and high dry matter; CMD susceptible, CBSD status unknown
Ex-ndolo	Landrace	High yields, mealiness and early maturing; CMD susceptible, CBSD status unknown
Ex-Mariakani	Landrace	High yields; CMD susceptible, CBSD status unknown

Figure 2: The process of establishing cassava stem cuttings in the glasshouse.

(A) Cassava stems planted in plastic pots, (B) Cassava stems covered with polythene bags to allow faster sprouting, (C) Two – week old plantlets, and (D) Fully established cassava plants six weeks after planting.

3.1.2 Source of cassava brown streak virus inoculum

Stems of susceptible cassava cultivar Dodoma exhibiting CBSD symptoms were collected from the Kenya Plant Health Inspectorate Service (KEPHIS), established in pots in the glasshouse and used as source of cassava brown streak virus inoculum (Figure 3). The presence of CBSD in established source of inoculum plants were confirmed by reverse transcriptase (RT)-PCR.

Figure 3: Cassava brown streak virus infected cultivar Dodoma used as the source of inoculum.

Source (own pictures)

3.1.2.1 RNA extraction

Extraction of RNA was carried out as delineated by Monger *et al.* (2001) with some modifications. The leaves of CBSD symptomatic cultivar Dodoma (0.5 g) were ground in 700 µl of the grinding buffer (2% CTAB, 1M Tris pH 8.0, 0.5 M EDTA and 5 M NaCl) warmed at 65 ºC. The suspension was placed in an Eppendorf tube and incubated for 30 minutes at 65 °C with gentle mixing by inversion after every 10 minutes. The samples were kept at room temperature

(23 ± 2º C) for 10 minutes. An equal volume (700 µl) of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion for 10 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes using a centrifuge (Mikro 200R model, Berlin, Germany) and the supernatant (550 µl) removed into a clean Eppendorf tube. The chloroform extraction was repeated and 490 µl of the supernatant transferred into a clean Eppendorf tube. The nucleic acid was precipitated in 0.7 volumes of ice-cold isopropanol at -20 ºC and shaken gently. The tubes were centrifuged at 10,000 rpm for 10 minutes. The isopropanol was carefully decanted and the nucleic acid washed in 500 µl of 70% ethanol. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4 ºC. The ethanol was decanted and the pellet air dried for 40 minutes then resuspended in 50 µl of RNAse free water. The quality and integrity of RNA was confirmed by agarose gel electrophoresis and visualized under UV light.

3.1.2.2 Synthesis of first strand cDNA

Bioneer Cycle Script Reverse Transcriptase cDNA synthesis kit was used for cDNA synthesis. In a sterile RNase free tube, 5 µl RNA, 1 µl of primer CBSV 10F and 5 µl of nuclease free water was added, mixed gently, incubated at 65 ºC for 10 minutes, chilled on ice, centrifuged briefly again for 2 minutes and placed on ice. To the reaction mix, 4 µl Cycle Script reaction buffer, 1 µl cycle script reverse transcriptase enzyme, 2 µl dNTP and 2 µl 100 mM DTT was added, mixed gently and centrifuged. The mix was incubated at 50 ºC for 1 hour. The reaction was terminated by heating at 90 ºC for 5 minutes and the tubes placed on ice for use in PCR.

3.1.2.3 Polymerase chain reaction

The synthesized cDNA was subjected to RT-PCR using primer set CBSV 10 (5'-ATCAGAA TAGTGTGACTGCTGG-3') and CBSV 11 (5'-CCACATTATTATCGTCACCAGG-3') which amplify ~230 bp length nucleotides. In a 25 µl PCR reaction volume, 1.5 mM MgCl, 10 µM of each primer, 2.5µl of 1x *Taq* polymerase buffer, 250 µM dNTPs mixture, 2.5 units of *Taq* DNA polymerase (Invitrogen), 2 µl of cDNA topped with nuclease-free water was premixed in a 1.5 ml PCR tube and subjected to PCR reaction. The reaction was run at 94 ºC for 2 minutes for the initial denaturation followed by reaction at 94 ºC for 1 minute, 52 ºC for 1 minute and 72 ºC for 1 minute, repeated for 35 cycles and a final extension at 72 ºC for 10 minute. The mixture was held at 4 ºC.

3.1.2.4 Agarose gel electrophoresis of RT-PCR products

Amplified RT-PCR products were analyzed using horizontal gel electrophoresis. The product (10 µl) was mixed with 6x gel loading dye (1 µl) and loaded onto an agarose gel (1.0% w/v) stained in ethidium bromide solution (0.2 µl) in 1 × TAE (Tris-Acetate- EDTA) buffer at 70 V for 65 minutes and visualized using the Gel Doc XR+ imaging system (Bio-Rad, New York, USA).

3.1.3 Graft inoculation of cassava cultivars with cassava brown streak virus-infected scions

For each cultivar, healthy plants were top-graft inoculated at 6 weeks after planting with CBSV-infected scions (from cassava cultivar Dodoma) as described by Anjanappa *et al*. (2016). A vertical slit was made on the stem of healthy plant using a sterile blade. A scion of the CBSV-infected plant was cut and inserted in the slit of the healthy plant such that the phloem and xylem of both plants coincided. Graft portions were taped tightly with parafilms to allow union. Grafted

plants were shielded from excessive evaporation by covering them with a polythene bag which was raised to avoid contact with the plant. After 10 days the polythene bags were removed, grafted-inoculated plants were kept in the glass house and monitored visually for development of CBSD symptoms. Control plants were mock graft-inoculated with disease-free scions of cultivar Dodoma.

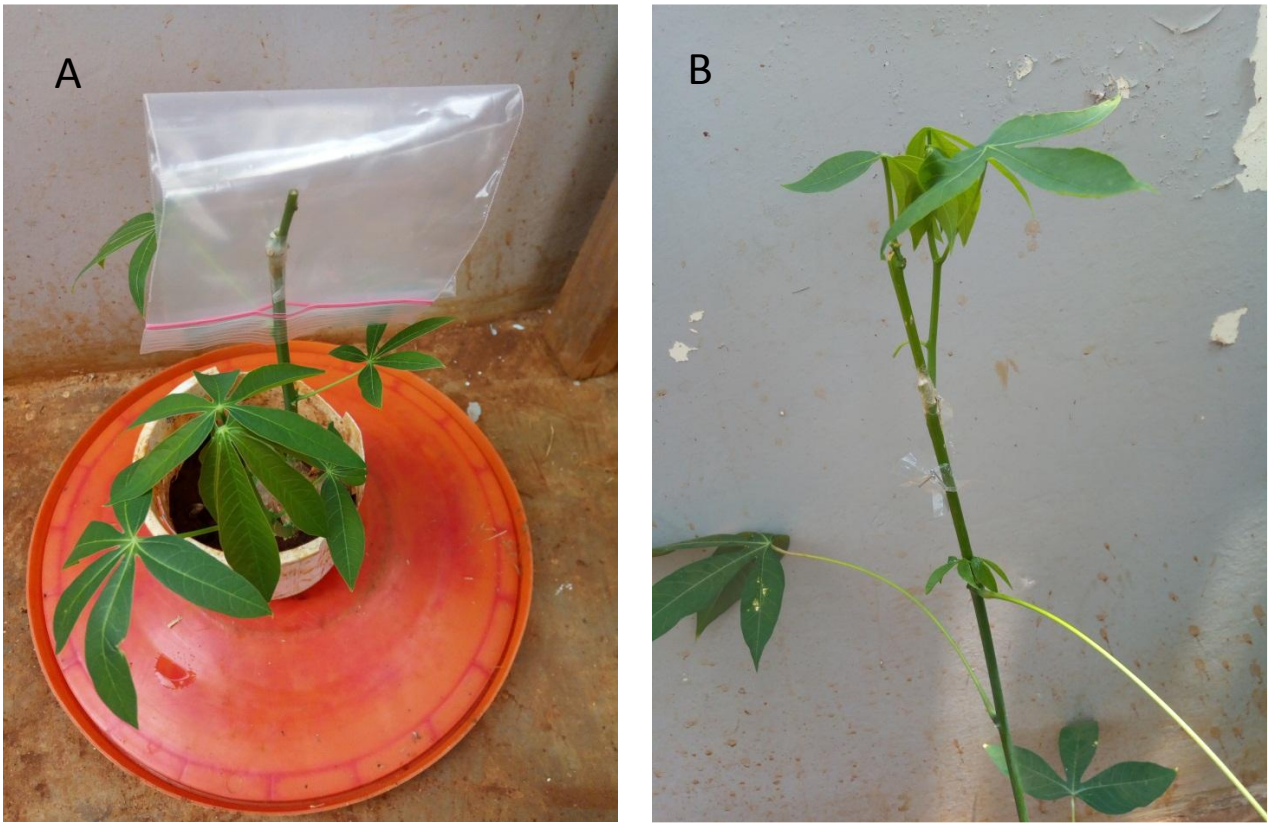

Figure 4: The procedure for grafting clean cassava plants with cassava brown streak virus infected scion of cultivar Dodoma.

(A) Insertion of the CBSV infected scion to the clean cassava cultivar, tapped with a parafilm and covered with a polythene bag. (B) Successful graft union

3.1.3.1 Symptoms of inoculated cassava plants

Development of symptoms on the inoculated cultivars was evaluated every seven days for a period of 12 weeks. Symptoms were scored using a scale of 1 – 5, where 1 = no visible CBSD symptoms, 2 = mild foliar symptoms on some leaves, 3 = pronounced foliar symptoms but no die-back, 4 = pronounced foliar symptoms which might include slight die-back of terminal branches, and 5 = severe foliar symptoms and plant die-back (Hillocks *et al*., 1996).

3.2 Confirmation of infection with cassava brown streak virus in inoculated test plants

The test plants inoculated with CBSV were confirmed positive to the infection using RT-PCR as described in section 3.1.2.1 to section 3.1.2.4

3.3 Determination of physiological and biochemical parameters of cassava plants upon inoculation with cassava brown streak virus

Symptomatic leaf samples from the CBSV inoculated cultivars were harvested at 8 wpi and at 12 wpi for analysis of physiological parameters, hydrogen peroxide, lipid peroxidation and antioxidant enzymes. The symptomatic leaf samples were stored at -20 ºC throughout the laboratory analysis.

3.3.1 Hydrogen peroxide and lipid peroxidation

The amount of hydrogen peroxide was determined by measuring the optical density using potassium iodide as described by Velikova *et al*. (2000). Exactly 0.5 g leaf sample was homogenized with a mortar and pestle in 2 ml of ice cold 0.1% (w/v) trichloroacetic acid (TCA). The suspension was centrifuged at 10,000 rpm for 30 minutes at 4 ºC using centrifuge (Mikro

200R model, Berlin, Germany). To 0.5 ml of the supernatant, 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide was added. The absorbance was read at 390 nm using a spectrophotometer (UV-mini 1240, Kyoto, Japan) and amount of H_2O_2 calculated using the extinction co-efficient 0.28/ mM/cm and expressed as μmol/g FW (fresh weight).

Lipid peroxidation was estimated by the amount of malondialdehyde produced using the method described by Hodges *et al*. (1999) and Chen and Gallie (2006) based on thiobarbituric acid (TBA). "In 4 ml of 0.1% (w/v) trichloroacetic acid (TCA) solution on ice, 0.2 g leaf sample was homogenized. An additional 1 ml of TCA was added to the homogenate in the Eppendorf tube. The suspension was centrifuged at 10,000 rpm for 5 minutes and 0.5 ml of the supernatant collected. To the supernatant, 1ml of 20% (w/v) TCA containing 0.5% (w/v) TBA was added and incubated at a water bath at 95 ºC for 30 minutes and quickly cooled on ice. The mixture was centrifuged at 10,000 rpm for 10 minutes and the absorbance measured at 532 and 600 nm. Non-specific turbidity was corrected by subtracting the absorbance at 600 nm from that at 532 nm". Malondialdehyde (MDA) concentration was calculated with its extinction coefficient 155 mM^{-1} cm^{-1} and expressed as nmol malondialdehyde g^{-1} fresh mass using the formula:

MDA content = Amount of extraction buffer (ml) x Amount of supernatant (ml) x[(Abs $_{532}$ – Abs $_{600}$)/155] × 1000/Amount of sample

Where 532 nm = maximum absorbance of the TBA-MDA complex; 600 nm = the correction for non-specific turbidity and 155 $mM^{-1}cm^{-1}$ = specific molar extinction coefficient for MDA.

3.3.2 Total chlorophyll content

The amounts of chlorophyll a (Chl a); b (Chl b) and total (Chl T) were determined spectrophotometrically as described by Upadhyaya *et al.* (2010). Leaves weighing 0.1 g from each treatment were placed in Eppendorf tubes and in 1 ml of 80% (v/v) acetone added to the leaves. The samples were incubated in the dark overnight. The supernatant absorbance was recorded at wavelengths of 647 and 664 nm using a spectrophotometer (UV–mini 1240, Kyoto, Japan). Chl a, Chl b and Chl T was calculated using the equations:

Chl a = $11.94A_{664} - 1.93A_{647}$, Chl b = $20.36A_{647} - 5.50A_{664}$, and chl T = Chl a + Chl b. The chlorophyll content was expressed in μg g^{-1} FW.

3.4 Determination of antioxidant enzymes in cassava cultivars following inoculation with cassava brown streak virus

3.4.1 Ascorbate peroxidase, catalase and peroxidase enzymes

Each symptomatic leaf sample from the CBSV inoculated plants weighing 0.5 g was homogenized using a pestle and mortar in 2 ml of buffer (1% w/v polyvinylpyrolydone (PVP), 0.2 mM EDTA and 100 mM potassium phosphate buffer pH (6.8)) on ice. The mixture was centrifuged at 10,000 rpm for 20 minutes at 4 ºC. The supernatants were assayed for catalase, peroxidase and ascorbate peroxidase enzyme activities.

Ascorbate peroxidase (APX) activity was assayed by checking the oxidation of ascorbic acid and recording the change at an absorbance of 290 nm. The leaf extract of 10 µl was mixed with 1 ml of a reaction buffer containing 0.25 mM ascorbic acid, 0.2 mM Tris/HCl buffer (pH 7.8), and 0.5

mM H_2O_2. The ascorbate peroxidase activity was calculated from the extinction coefficient (2.8 mM^{-1} cm^{-1}) ascorbate (Nakano and Asada, 1981).

Catalase (CAT) activity was determined as described by Cakmak *et al*. (1993). The reaction buffer (3ml) containing 15 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0) was added to 50 µl of the enzyme extract. The activity of catalase was measured as the decrease in absorbance at 240 nm for 1 minute due to the decomposition of H_2O_2. The activity was calculated from the extinction coefficient (40 mM^{-1} cm^{-1}) for H_2O_2.

Peroxidase (POD) activity was determined by adding 50 µl of leaf homogenate to 2 ml of the reaction mixture that contains 25 mM H_2O_2, 25 mM guaiacol and 50 mM sodium acetate buffer (pH 7). Peroxidase activity was determined by recording the absorbance at 470 nm which increases due to formation of tetra guaiacol (coefficient of extinction 26.6 mM^{-1} cm^{-1}).

3.5 Data analysis

The data on physiological parameters and antioxidant enzymes were recorded for statistical analysis. The data were subjected to analysis of variance using GenStat software version 15. Comparisons of the means were done using Tukey HSD test at 5% probability level.

CHAPTER FOUR: RESULTS

4.1 Detection of cassava brown streak virus in plants used as the inoculum by RT-PCR

Cultivar Dodoma which was used as the source of CBSV inoculum and the inoculated test plants tested positive for the virus after carrying out RT-PCR amplification of isolated RNA. The expected RT-PCR amplified products of 230 bp were obtained hence the presence of CBSV confirmed in the plants (Figure 2).

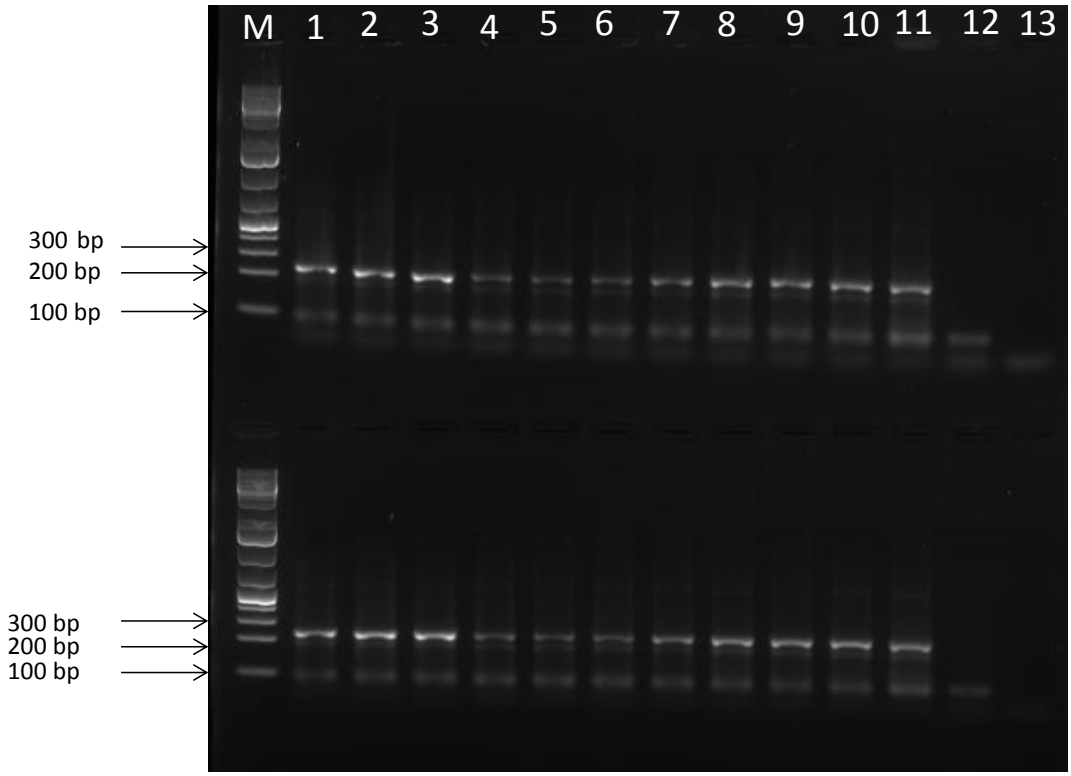

Figure 5: Agarose gel electrophoresis of RT-PCR amplified fragments with primers specific for CBSV.

Lane (M) 1 kb molecular weight marker, Lanes (1-10) CBSV-infected plants of cassava cultivars TME 14, Ebwanatereka 1, Ebwanatereka 2, Karembo, Tajirika, Karibuni, TME 204, Ex-ndolo, TME 14 and Kibandameno respectively, Lane (11) Positive control (CBSV-infected plant of cultivar Dodoma), Lane (12) Virus-free cassava cultivars Karibuni and Lane (13) Sterile double distilled water as non-template control.

4.2 Response of cassava cultivars to inoculation with cassava brown streak virus

All the cassava cultivars exhibited symptoms of cassava brown streak disease on their leaves after inoculation with the virus but with varying degrees of severity. The plants expressed yellow vein banding mainly on the lower, older leaves and feathery chlorosis along secondary and tertiary veins (Figure 6). Cultivars Karibuni, Karembo and Tajirika expressed the foliar symptoms at 8 wpi and the chlorosis was mild therefore having a score of 2 (Figure 6). Cultivars TME 7 and TME 204 expressed pronounced foliar symptoms at 6 wpi but did not have any die-back hence had a score of 3 on the severity scale. Cultivars Ex-ndolo, MM96/7151, TME 14, Ebwanatereka 1 and Ebwanatereka 2 expressed their foliar symptoms at 4 wpi with slight dieback at 12 wpi and were rated 4 on the virus score scale (Figure 7). Cultivars Kibandameno, Ex-Mariakani and KME-1 expressed foliar symptoms as early as at 4 wpi, and at 12 wpi; the three cultivars had severe chlorosis and die- back were therefore assigned a score of 5. Cultivars Karibuni, Karembo and Tajirika were rated as tolerant. Cultivars Ebwanatereka 1, Ebwanatereka 2, Kibandameno, TME 14, TME 7, TME 204, Ex-Mariakani, Ex-ndolo, MM96/7151 and KME-1 were rated as susceptible to CBSD (Figure 6).

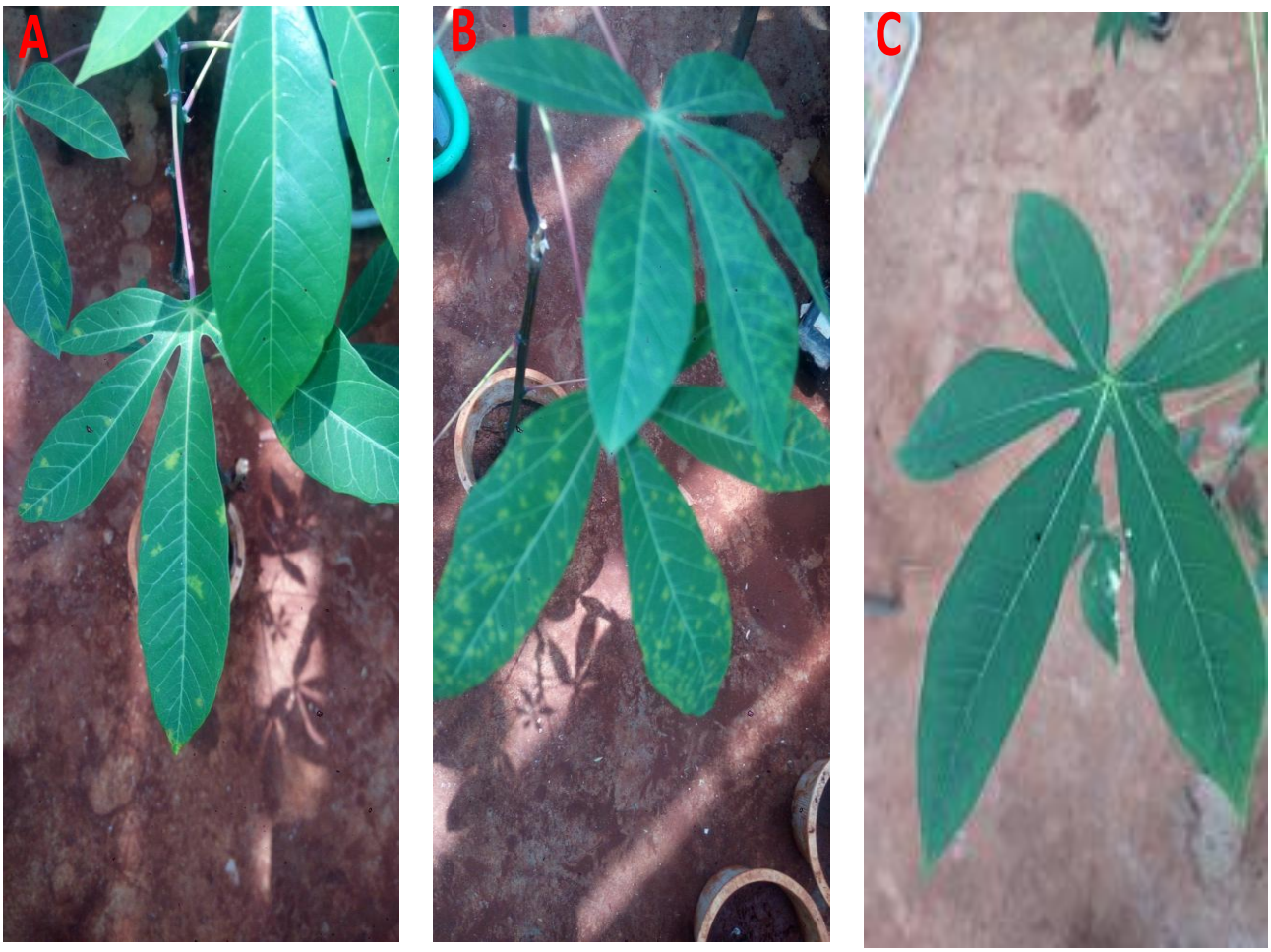

Figure 6: Cassava brown streak disease symptoms on cassava leaves inoculated with CBSV.

(A) Tolerant cassava cultivar Karembo, (B) susceptible cassava cultivar Ebwanatereka 1 and (C) mock-inoculated cassava.

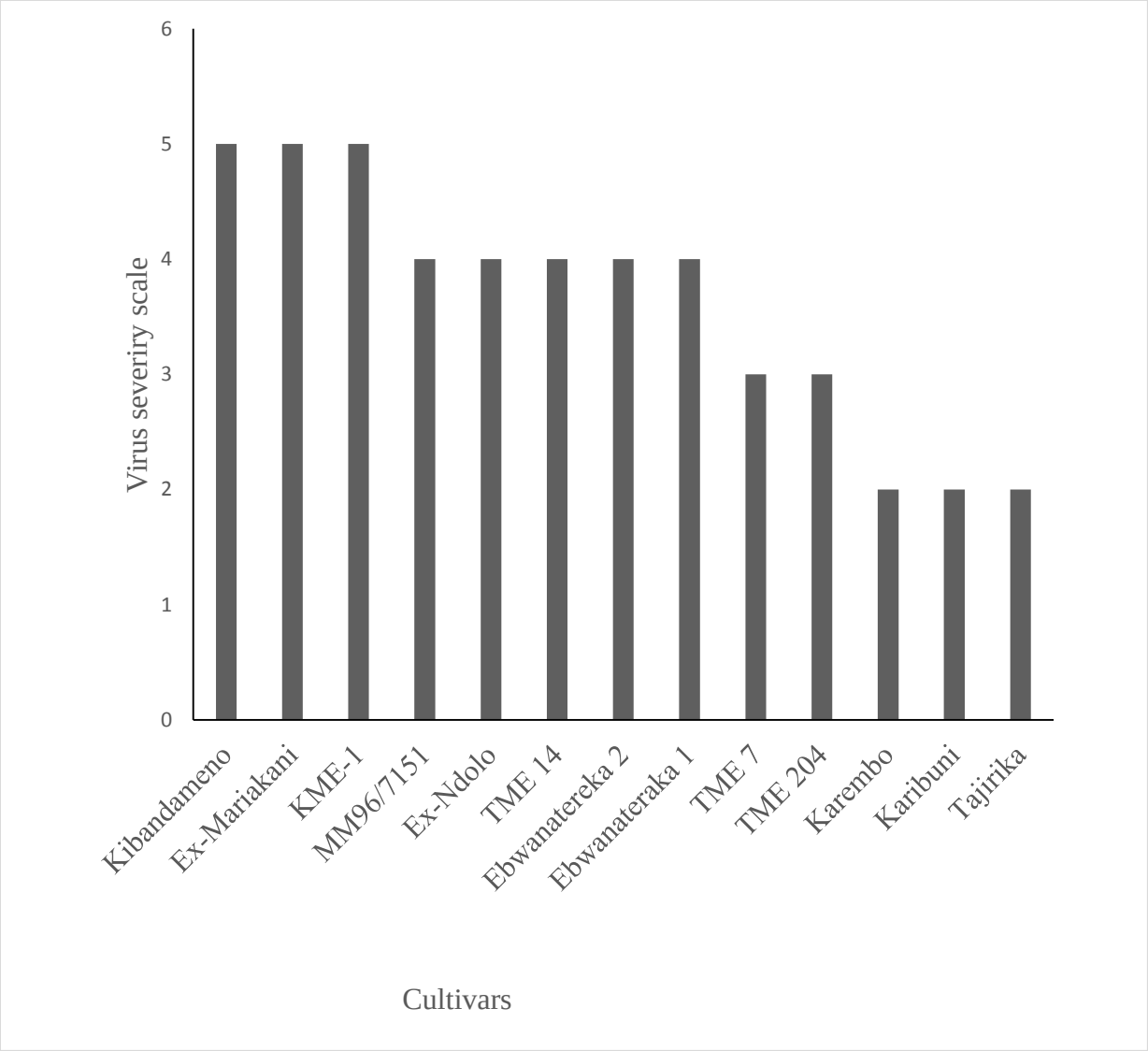

Figure 7: Virus score of susceptibility of various cassava cultivars to cassava brown streak virus infection**.**

Where: "1 = no visible CBSD symptoms, 2 = mild foliar symptoms on some leaves, 3 = pronounced foliar symptoms but no die-back, 4 = pronounced foliar symptoms which might include slight die-back of terminal branches, and 5 = severe foliar symptoms and plant die-back" (Hillocks *et al*., 1996).

4.3 Physiological parameters

4.3.1 Chlorophyll content in leaves of cassava cultivars inoculated with cassava brown streak virus

The amount of chlorophyll was significantly different ($P \leq 0.05$) among all inoculated and non-inoculated plants of all cultivars at 8 weeks post inoculation (wpi). Pairwise comparison of inoculated and non-inoculated plants showed a significant decrease ($P \leq 0.05$) in chlorophyll content in the inoculated plants of cultivars Ex-Mariakani, KME-1 and TME 14 compared to their non-inoculated counterparts. However, inoculated plants of cultivars Ex-ndolo, MM96/7151, Karibuni, Tajirika, TME 204, TME 7, Karembo, Ebwanatereka 1, Ebwanatereka 2 and Kibandameno showed no significant decrease ($P \geq 0.05$) in the amount of chlorophyll when compared to their non-inoculated counterparts. At 12 wpi, inoculated plants of cultivars Ebwanatereka 1, TME 7, TME 14, TME 204, MM96/7151, KME-1, Ex-ndolo, Ex-Mariakani and Ebwanatereka 2 showed the greatest reduction in chlorophyll content. Inoculated plants of cultivars Karibuni, Karembo, Tajirika and Kibandameno did not show significant decline ($P \geq 0.05$) in the amount of chlorophyll when compared to the non-inoculated counterparts (Table 1). At 8 wpi the inoculated plants of susceptible cultivars had a 14.14% reduction in chlorophyll compared to a 7.43 % reduction in chlorophyll content in inoculated plants of the tolerant cultivars. At 12 wpi, there was a 20.69 % reduction in chlorophyll in inoculated plants of susceptible cultivars compared to 9.38 % reduction in plants of tolerant cultivars.

Table 2: The amount of chlorophyll (µg/g FW) in inoculated and non-inoculated cassava cultivars at 0, 8 and 12 weeks post inoculation with CBSV.

Cultivars	CBSV infection	Amount of chlorophyll (µg/g FW) at different times (weeks)		
		0	8	12
Ebwana 1	(+)	12.43 hi	10.79 k	8.55 j
	(-)	12.83 hi	14.2 h-k	12.28 hi
Ebwana 2	(+)	26.91 a-c	22.07 b-f	18.26 ef
	(-)	26.87 a-c	24.04 a-d	23.06 a-c
Ex-Mariakani	(+)	23.83 b-d	20.59 d-g	16.33 fg
	(-)	23.23 c-e	25.04 a-c	23.86 ab
Ex-ndolo	(+)	20.11 e-g	18.59 fg	17.43 ef
	(-)	20.0 e-g	21.61 c-f	21.16 cd
TME 204	(+)	16.72 f-h	14 h-k	13.95 gh
	(-)	16.41 g-i	16.67 g-i	17.39 ef
Karibuni	(+)	12.2 hi	11.6 k	10.76 ij
	(-)	12.44 hi	12.38 i-k	11.11 i
Kibandameno	(+)	28.82 a	25.67 a-c	21.87 a-c
	(-)	28.48 ab	28.19 a	24.06 a
KME-1	(+)	26.5 a-c	19.08 e-g	17.6 ef
	(-)	26.18 a-c	23.47 b-d	21.57 b-d
MM96/7151	(+)	16.8 f-h	16.31 g-i	11.37 i
	(-)	16.72 f-h	17.89 f-h	17.69 ef
Tajirika	(+)	11.61 i	12.35 jk	10.84 ij
	(-)	11.88 hi	13.51 i-k	12.26 hi
TME 14	(+)	21.4 d-f	20.17 d-g	16.06 fg
	(-)	21.17 e-g	24.97 a-c	19.18 de
Karembo	(+)	12.46 hi	11.55 k	10.29 ij
	(-)	12.55 hi	12.45 i-k	11.82 hi
TME 7	(+)	26.63 a-c	23.37 b-d	19.13 de
	(-)	26.92 a-c	25.92 ab	22.24 a-c
LSD ($P \leq 0.05$)		2.564	2.241	1.2989
CV (%)		9.2	8.5	5.6

The data represents means, LSDs and % CV of the amounts of chlorophyll. Means accompanied by the same letter (s) in each column are not significantly different according to Tukey HSD test ($P \leq 0.05$). LSD – Least significant difference, CV– Coefficient of variation, (+) – inoculated, (-) – non-inoculated

4.3.2 Changes in hydrogen peroxide and malondialdehyde content in cassava plants inoculated with cassava brown streak virus

The amount of hydrogen peroxide (H_2O_2) was significantly different ($P \leq 0.05$) in all inoculated and non-inoculated plants at 8 wpi. Pairwise comparison of inoculated and non-inoculated plants showed a significant increase in the amount of H_2O_2 in inoculated plants of cultivars Karibuni, Ex-ndolo, Ebwanatereka 2, TME 7, TME 204 and TME 14 in comparison to their non-inoculated counterparts. Inoculated plants of cultivars Ebwanatereka 1, Ex-Mariakani, KME-1, MM96/7151, Kibandameno, Tajirika and Karembo did not show any significant increase ($P \geq 0.05$) in the amount of H_2O_2 when compared to their non-inoculated counterparts. At 12 wpi, there was a significant difference in the amount of H_2O_2 in inoculated plants of all cultivars compared to the non-inoculated controls. Inoculated plants of cultivars Karibuni, Tajirika, TME 14 and Karembo had the highest amount of H_2O_2, whereas inoculated plants of cultivars KME-1, Ex-Mariakani, Ebwanatereka 2 and MM96/7151 had the lowest amounts of H_2O_2. Pairwise comparison of inoculated and non-inoculated plants of all the cultivars showed no significance ($P \geq 0.05$) difference in the amount of H_2O_2.

There was a significant difference ($P \leq 0.05$) in the amount of malondialdehyde (MDA) in all the virus inoculated and non-inoculated plants at 8 wpi. Pairwise comparison of inoculated and non-inoculated plants of all cultivars yielded significant increase in MDA in inoculated plants of cultivars Ex-Mariakani, TME 204, MM96/7151 and TME 14 compared to the non-inoculated counterparts, On the other hand, inoculated plants of cultivars Karibuni, Karembo, Tajirika, TME 7, Ebwanatereka 1, Ebwanatereka 2, KME-1, Ex-ndolo and Kibandameno did not exhibit significant increase ($P \geq 0.05$) in the amount of MDA compared to their non-inoculated

counterparts. There was also a significant difference ($P \leq 0.05$) in the amount of MDA among all plants of the virus inoculated and non-inoculated cultivars at 12 wpi. Pairwise comparison of inoculated and non-inoculated plants showed no significant ($P \geq 0.05$) increase in MDA in inoculated plants of cultivars Karibuni, Tajirika, Karembo and Ebwanatereka 1. Inoculated plants of cultivars Ebwanatereka 2, Ex-ndolo, TME 14, TME 204, TME 7, Kibandameno, KME-1, Ex-Mariakani and MM96/7151 exhibited significant increases in amount of MDA compared to their non-inoculated counterparts. Inoculated plants of cultivars Kibandameno, KME-1, TME 7 and Ex-ndolo had the highest amounts of MDA, whereas Karibuni, Tajirika, Karembo and Ebwanatereka 1 had the lowest amounts of MDA (Table 2).

Table 3: The amount of hydrogen peroxide and malondialdehyde (nmol /g FW)) in inoculated and non-inoculated cassava cultivars at 0, 8 and 12 weeks post inoculation with CBSV.

Cultivars	CBSV infection	Amount of H_2O_2 (nmol/g FW) at different times (weeks)			Amount of MDA (nmol/g FW)at different times (weeks)		
		0	8	12	0	8	12
Ebwana 1	(+)	219.4 a-d	260.2e	225.2a-c	3.8a	1.90g	2.23d
	(-)	221.5 a-d	240.5ef	205.5a-c	3.84a	1.71g	2.98d
Ebwana 2	(+)	146.1 de	407.1a	140.3a-c	2.69ab	6.6ab	16.02b
	(-)	145.2 de	178.6f-h	220.3a-c	2.81ab	5.27b-d	2.69d
Ex-Mariakani	(+)	145.9 de	259.2e	122.7bc	2.42ab	7.24a	18.26ab
	(-)	147.8 de	236.5ef	222.8a-c	2.69ab	2.59e-g	6.63cd
Ex-ndolo	(+)	244.2 abc	266.9c-e	220.7a-c	1.71ab	5.6b-d	21.1ab
	(-)	246.7 ab	177.8f-h	301.1a	1.92ab	3.88d-f	4.63d
TME 204	(+)	209.8 a-d	345ab	256.1ab	2.03ab	6.57ab	23.05ab
	(-)	208 a-d	184.2f-h	260.3ab	1.98ab	4.11de	1.63d
Karibuni	(+)	192.3 a-d	236.5ef	299.8a	1.32ab	1.31g	4.66d
	(-)	189.8 a-d	145.2g-i	224a-c	1.32ab	1.13g	4.84d
Kibandameno	(+)	246.5 a-c	327.8bc	179.6a-c	1.42ab	5.5b-d	25.63a
	(-)	246.9 ab	193.8fg	147.1a-c	1.48ab	4.58cd	2.42d
KME-1	(+)	213.4 a-d	326.1b-d	59.4c	1.84ab	2.58e-g	24.15ab
	(-)	212.3 a-d	268.4c-e	122.8bc	1.98ab	1.95g	2.03d
MM96/7151	(+)	103.6 e	128.6hi	161.9a-c	0.94b	7.18a	17.87ab
	(-)	103.8 e	96.3i	161.7a-c	1.0ab	1.55g	4.24d

Tajirika	(+)	189.2 a-d	231.7ef	297.3a	1.31ab	2.03g	3.36d
	(-)	191.9 a-d	220.2ef	186.3a-c	1.11ab	1.45g	1.63d
TME 14	(+)	167.5 b-e	351.5ab	279ab	3.45ab	6.18a-c	15.40bc
	(-)	169 b-e	149.4g-i	198a-c	3.89a	2.23fg	4.42d
Karembo	(+)	258.6 a	275c-e	262.5ab	2.02ab	2.61e-g	5.71d
	(-)	261.9 a	261.9de	239.2ab	2.13ab	1.39g	4.19d
TME 7	(+)	164.8 b-e	263.6c-e	132.1a-c	2.57ab	4.71cd	22.98ab
	(-)	162.1 c-e	180.9f-h	205.2a-c	2.84ab	4.1de	4.31d
LSD ($P \leq 0.05$)		44.08	65.16	90.6	1.509	1.801	4.64
CV (%)		16.3	19.4	31.4	49.3	34.7	34.7

The data represents means, LSDs and % CV of the amounts of H_2O_2 and MDA. Means accompanied by the same letter (s) for each column are not significantly different according to Tukey HSD test ($P \leq 0.05$). LSD – Least significant difference, CV – Coefficient of variation, H_2O_2 – hydrogen peroxide, MDA – malondialdehyde, (+) – inoculated, (-) – non-inoculated.

4.4 Antioxidant enzyme response of cassava cultivars inoculated with cassava brown streak virus

4.4.1 Ascorbate peroxidase enzyme

There was a significant difference ($P \leq 0.05$) in the amount of ascorbate peroxidase (APX) between all plants of inoculated and non-inoculated cultivars at 8 wpi. Pairwise comparison of inoculated and non-inoculated plants showed a significant increase ($P \leq 0.05$) in the amount of APX in inoculated plants (6.85 µmol/g FW) of cultivar Tajirika when compared to non-inoculated Tajirika (2.48 µmol/g FW). On the other hand, inoculated plants of cultivars Karibuni, Karembo, Ebwanatereka 1, Ebwanatereka 2, MM96/7151, Ex-ndolo, Ex-Mariakani, TME 204, TME 7, TME 14, KME-1 and Kibandameno exhibited no significant increase ($P \geq 0.05$) in the amount of APX when compared to their non-inoculated counterparts. At 12 wpi, the amount of APX was significantly different ($P \leq 0.05$) among inoculated and non-inoculated plants of all cultivars. Pairwise comparison of inoculated and non-inoculated plants showed a significant increase ($P \geq 0.05$) in the amount of APX in the inoculated plants of cultivars Karibuni, Karembo, Tajirika and Ebwanatereka 2. Inoculated plants of cultivars Ebwanatereka 1, MM96/7151, Ex-ndolo, Ex-Mariakani, TME 204, TME 7, TME 14, KME-1 and Kibandameno did not show significant increase ($P \geq 0.05$) in the amount of APX when compared to their non-inoculated counterparts (Table 3).

4.4.2 Catalase enzyme

There was a significant difference ($P \leq 0.05$) in the amount of catalase (CAT) among plants of inoculated and non-inoculated cultivars at 8 wpi. Pairwise comparison of inoculated and non-

inoculated plants of all cultivars yielded no significant differences in the amount of CAT. However, at 12 wpi there was a significant increase ($P \leq 0.05$) in the amount of CAT in inoculated plants of all cultivars compared to the non-inoculated plants. Cultivar TME 7 had the highest increment (3.44 µmol/g FW) in the amount of CAT at 12 wpi (Table 3).

4.4.3 Peroxidase enzyme

At 8 wpi, there was a significant ($P \leq 0.05$) difference in the amount of peroxidase (POD) among all plants of inoculated and non-inoculated cultivars. Pairwise comparison of inoculated and non-inoculated plants showed a significant increase in the amount of POD in the inoculated plants of cultivars Karibuni, Karembo, TME 204 and Tajirika. On the other hand, plants of inoculated cultivars Ebwanatereka 1, MM96/7151, Ex-ndolo, Ex-Mariakani, Ebwanatereka 2, TME 7, TME 14, KME-1 and Kibandameno did not exhibit significant differences ($P \geq 0.05$) in the amount of POD when compared to the non-inoculated counterparts. At 12 wpi, there was also a significant difference ($P \leq 0.05$) in the amount of POD among all plants of the inoculated and the non-inoculated cultivars. Again, plants of inoculated cultivars Karibuni, Tajirika and Karembo had higher amount of POD than their controls, whereas plants of inoculated cultivars TME 204, Ebwanatereka 1, MM96/7151, Ex-ndolo, Ex-Mariakani, TME 204, TME 7, TME 14, KME-1 and Kibandameno did not exhibit significant increase ($P \geq 0.05$) in the amount of POD when compared to their non-inoculated (Table 3).

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Table 4: The amount of catalase, ascorbate peroxidase and peroxidase enzymes (µmol/g FW) in inoculated and non-inoculated cassava cultivars at 0, 8 and 12 weeks post inoculation with CBSV.

Cultivars	CBSV infection	Amount of CAT (µmol/g FW) at different times (weeks)			Amount of APX (µmol/g FW) at different times (weeks)			Amount of POD (µmol/g FW) at different times (weeks)		
		0	8	12	0	8	12	0	8	12
Ebwana 1	(+)	0.67ab	1.38ab	1.71 cde	1.89def	2.18c	2.68 def	0.65de	1.36cde	1.17 d
	(-)	0.69ab	0.34b	0.61fg	1.89def	1.69bc	2.28def	0.64de	0.81e	0.79d
Ebwana 2	(+)	1.86ab	2.04ab	2.44bc	3.87a	5.26abc	6.85ab	1.16cde	1.50bcd	1.41cd
	(-)	1.91a	0.94ab	0.39g	3.77a	4.96abc	3.77def	1.09cde	1.28de	1.20d
Ex-Mariakani	(+)	1.41ab	1.61ab	2.71ab	1.79def	3.37abc	2.78def	0.83de	1.34cde	1.17d
	(-)	1.39ab	0.28b	0.77fg	1.59ef	1.19c	2.58def	0.82de	1.13de	1.086d
Ex-ndolo	(+)	0.85ab	1.4ab	2.66ab	2.08def	3.37abc	4.56bcd	1.77abc	2.74a	2.55a
	(-)	0.86ab	0.21b	0.56g	2.08def	1.09c	2.48def	1.76abc	2.63a	2.42ab
TME 204	(+)	0.74ab	1.23ab	2.66ab	1.39f	3.47abc	3.97def	2.11ab	1.51bcd	1.43cd
	(-)	0.73ab	0.19b	0.48g	1.49f	1.98c	2.18def	2.09ab	0.79e	0.87d
Karibuni	(+)	0.45ab	1.09ab	2bcd	1.98def	4.86abc	6.75abc	0.78de	2.67a	2.42ab
	(-)	0.47ab	0.31b	0.54g	1.89def	1.79c	1.89ef	0.75de	0.84de	0.80d
Kibandameno	(+)	0.98ab	1.67ab	2.48bc	1.89def	3.47abc	4.27cde	1.48bcd	2.67a	2.51a
	(-)	1.09ab	1.28ab	0.93efg	1.79def	1.79 c	2.88def	1.48bcd	2.08abc	2.24abc
KME-1	(+)	0.66ab	1.53ab	1.61de	1.49f	3.77abc	3.97def	2.58a	2.17ab	2.17abc
	(-)	0.68ab	0.28b	0.43g	1.59ef	1.59c	1.98ef	2.59a	1.59bcd	1.59bcd

MM96/7151	(+)	1.31ab	1.83ab	2.48bc	2.68bcd	3.27abc	4.07def	1.72abc	2.62a	2.54a
	(-)	1.26ab	0.24b	0.56g	2.58cde	2.28bc	3.67def	1.73abc	2.46a	2.17abc
Tajirika	(+)	0.81ab	1.35ab	2.41bc	2.58cde	6.85a	7.44a	0.39e	2.42a	2.17abc
	(-)	0.79ab	0.64ab	0.69fg	2.58cde	2.48bc	1.69f	0.39e	0.88de	0.79d
TME 14	(+)	1.31ab	1.88ab	2.72ab	3.67ab	4.46abc	3.08def	0.66de	1.47bcd	1.22d
	(-)	1.37ab	1.18ab	1.38def	3.47abc	2.08c	2.88def	0.66de	1.09de	0.97d
Karembo	(+)	0.34b	0.88ab	1.8cd	1.98def	6.45ab	7.64a	0.99cde	2.46a	2.26bc
	(-)	0.36b	0.2b	0.55g	2.08def	2.98abc	3.18def	0.98cde	1.09de	0.92d
TME 7	(+)	1.44ab	2.86a	3.44a	2.08def	3.87abc	3.37def	0.49e	1.42cde	1.38cd
	(-)	1.43ab	1.13ab	0.93efg	1.98def	1.69c	2.88def	0.46e	1.21de	1.17 d
LSD (P ≤ 0.05)		0.8	1.31	0.8	1.08	2.26	1.31	0.46	0.39	0.48
CV (%)		57.4	86.4	36.9	34.2	50.7	25.2	27	16.5	21.3

The data represents means, LSDs and % CV of the amounts of CAT, APX and POD. Means accompanied by the same letter (s) for each column are not significantly different according to Tukey HSD test (P ≤ 0.05). LSD – Least significant difference, CV – Coefficient of variation, CAT – catalase, APX – ascorbate peroxidase, POD – peroxidase, (+) – inoculated, (-) – non-inoculated.

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Response of cassava cultivars to inoculation with cassava brown streak infection

In the present study, there was variability in resistance and susceptibility of cassava cultivars to cassava brown streak virus (CBSV) challenge. Foliar symptoms of cassava brown streak virus were exhibited in all the cultivars graft-inoculated with CBSV but with varying degrees of severity. This suggested variation in response of the different cultivars to cassava brown streak disease (CBSD). The symptoms recorded were yellow vein banding along secondary and tertiary veins (feathery chlorosis) on the lower older leaves and chlorosis on the lamina similar to the CBSD symptoms observed in previous studies (Hillocks *et al*., 2001; Alicai *et al*., 2007; Irungu 2011; Mohammed *et al*., 2012). In this study, out of the thirteen cassava cultivars screened for resistance, only three were tolerant to CBSD. The three tolerant cultivars were Karembo, Tajirika and Karibuni. The tolerant cultivars showed mild leaf chlorosis on the leaves with the onset of symptoms occurring late at 8 weeks post inoculation (wpi) compared to the 4 wpi onset of symptoms in susceptible cultivars. There was no plant die-back in the tolerant cultivars. These disease symptoms in the tolerant varieties were similar to disease symptoms recorded in tolerant cultivar Kiroba by Patil *et al*. (2015) and in tolerant cultivars reported by Alicai *et al*. (2016).

Susceptible cultivars showed different degrees of severity hence the different rating on the severity score. In cultivars TME 7 and TME 204, the symptoms recorded were pronounced leaf chlorosis with no plant die-back and the onset of symptoms was at 6 wpi. In cultivars Ex-ndolo, MM96/7151, TME 14, Ebwanatereka 1 and Ebwanatereka 2 the symptoms expressed were pronounced leaf chlorosis accompanied by slight die-back with the onset of symptoms taking place 4 wpi. In the highly susceptible cultivars Kibandameno, Ex-Mariakani and KME-1, the

symptoms expressed were lots of yellow vein banding on the foliar and plant die-back with the onset of symptoms taking place as early as at 4 wpi. These disease symptoms on the susceptible cultivars relate to the disease symptoms observed by Hillocks *et al*. (2001) and Anjanappa *et al*. (2016) on cassava cultivars highly susceptible to CBSV.

5.2 Changes in chlorophyll content in cassava inoculated with cassava brown streak virus

Cassava cultivars inoculated with the cassava brown streak virus showed significant reduction in chlorophyll content. Cassava cultivars tolerant to CBSD namely Karibuni, Karembo and Tajirika expressed mild yellowing on the leaves thus recorded no significant reduction in the amount of chlorophyll. However, highly susceptible cultivars such as TME 7, MM96/7151, KME-1 Kibandameno, Ex-ndolo, Ex-Mariakani and Ebwana 2 exhibited a lot of chlorosis and yellowing in the leaves hence the remarkable reduction in the amounts of total chlorophyll. These findings agree with the findings of Guo *et al.* (2005) and Mofunanya *et al.* (2014) whereby the amount of chlorophyll reduced upon infection of mustard and pumpkin with turnip mosaic virus and Telfairia mosaic virus, respectively. Soybean susceptible to yellow mosaic virus showed greater reduction in chlorophyll content than the resistant soybeans when inoculated with the yellow mosaic virus (Kaur, 2016).

When plants are attacked by pathogens, the biosynthesis of compounds related to defense are upregulated while other growth related activities are reduced therefore allowing a decline in the rate of photosynthesis until the growth of pathogens is halted (Garavaglia *et al.*, 2010). Bilgin *et al.* (2010) reported that biotic damage to plant foliage caused down regulation of genes responsible for photosynthesis. This down regulation was particularly more pronounced in genes necessary for pigment synthesis. The most common symptom of plant viral infection is chlorosis

(Zhao *et al*., 2016). Similarly, cassava brown streak virus causes damage to leaves and one of the earliest and most visible symptoms of CBSD in cassava is chlorosis that appears in a feathery pattern, first along the margins of the secondary veins, later affecting tertiary veins and may develop into chlorotic blotches of older lower leaves (Nuwamanya *et al*., 2015). This yellowing of older cassava leaves caused by CBSD can be distinguished from that of normal plant senescence by presence of the green patches that occur in CBSD infected cassava (Irungu, 2011).

Morphological damage in the leaf upon viral infection exaggerates the physiological leaf damage leading to photo-damage of the chlorophyll photosystems (Nuwamanya *et al*., 2017). When viruses infect plants, they use the plant machinery system to replicate. They associate with the chloroplast to help in viral uncoating which is a vital step during viral replication (Zhao *et al*., 2016). Photosystem II is the first link in the chain of photosynthesis. When viral proteins associate with photosystem II during replication, the efficiency of this photosystem declines therefore lowering the rate of photosynthesis (Synková *et al*., 2006).

Alternatively, viruses could alter the efficiency of photosystem II by hindering the repair of D1 protein by the plant cell. D1 proteins are proteins integrated into the thylakoids of the chloroplast to form the core reaction center for photosystem II. The proteins are prone to photo-damage in excess light and must therefore be constantly degraded and replaced with a synthesized copy through photosystem II repair system to sustain photosynthesis (Che *et al*., 2013). Upon integration of viruses into the chloroplast, virus hinder repair of the D1 proteins since the plant cell synthesizes viral proteins at the expense of the D1 protein. The result is an impaired photosystem II electron chain which greatly damages the photosynthetic apparatus impeding chlorophyll synthesis. This leads to chlorosis and yellowing of the foliar (Synková *et al*., 2006).

Cassava brown streak virus interferes with photosynthetic metabolism by reducing the amount of starch in the starch granules of the chloroplast thus reducing synthesis of sugars. This affects the downstream synthesis of vital components like proteins and pigments which depend on sugar for synthesis thus affecting the rate of photosynthesis (Nuwamanya *et al.,* 2017; Zhao *et al*., 2016). In some resistant host plant interactions, chloroplasts act as a defense mechanism against viral infection. For instance, calcium sensing receptor on chloroplasts is involved in stromal calcium ion transients which is responsible for R gene mediated defense and basal resistance (Zhao *et al*., 2016). In susceptible host plant interactions, some chloroplast factors are impounded by the virus to block defense and facilitate viral entry and replication.

5.3 Changes in the content of hydrogen peroxide in cassava cultivars inoculated with cassava brown streak virus

Cassava brown streak virus tolerant cultivars had lower levels of hydrogen peroxide (H_2O_2) while the susceptible cultivars had the highest levels of H_2O_2 at 8 wpi. The findings of this study are in agreement with the report by Quan *et al*. (2008) whereby H_2O_2 at low concentrations induced production of defense genes leading to tolerance, whereas at higher amounts, H_2O_2 triggered programed cell death. In previous studies by Hakmaoui *et al*. (2012), the amount of H_2O_2 increased upon infection of tobacco with pepper mild mottle virus. Hernandez *et al*. (2006) suggested that an increase in reaction of species (ROS) upon infection of susceptible peach with plum pox virus could contribute to symptom development and pathogenicity in susceptible plant-virus interactions. The increase in H_2O_2 in susceptible cultivars at 8 wpi in this study could have been as a result of insignificant increase in peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) enzymes which are involved in detoxification of H_2O_2. Increase in

(peroxidase) POD in the tolerant cultivars could probably have detoxified the H_2O_2 hence the reduced levels of H_2O_2. At 12 wpi, inoculated plants of tolerant cultivars showed increased levels of H_2O_2 while susceptible cultivars showed reduced levels of H_2O_2 than at 8 wpi. The results of this study are in line with the findings of Madhusudhan *et al*. (2009) whereby the level of H_2O_2 increased in tobacco and tomatoes resistant to tobamoviruses. Madhusudhan *et al*. (2009) suggested that H_2O_2 at high concentrations act as a signal molecule to trigger defense mechanisms which could explain the increase in H_2O_2 in cassava cultivars tolerant to CBSD. Keller *et al*. (1998) reported that during plant-pathogen interactions, overproduction of H_2O_2 and its accumulation promotes local defense response. However, an increase in H_2O_2, superoxide radicals and hydroxyl which are reactive oxygen species can either lead to hypersensitive response leading to programmed cell death or can alternatively induce systemic acquired resistance (Mateo *et al*., 2004). In this study, increase in amount of catalase (CAT) in CBSD susceptible cultivars at 12 wpi could have resulted to detoxification of H_2O_2 hence its reduction.

Production of ROS is the first line of defense activated by plants upon infection by pathogens (Sharma *et al.,* 2012). Accumulation of ROS plays two roles in plants infected by pathogens, ROS may promote programmed cell death or limit pathogenic growth since they are highly toxic (Hernández *et al*., 2016). During any ROS burst, H_2O_2 always acts as a signal molecule to elicit a series of molecular, biochemical, and physiological responses in plant cells. Hydrogen peroxide liberated in the initial stages of pathogen infection in plants leads to a cascade of events such as lignification and papillae formation that leads to the strengthening of the cell wall. Papillae play a vital role as a barrier that prevents penetration of pathogens into the plant (Sharma *et al.*, 2012).

5.4 Changes in malondialdehyde content in cassava cultivars inoculated with cassava brown streak virus

Lipid peroxidation is a defense response mechanism that occurs during non-host interaction of plants with pathogens and can be used to detect oxidative damage. Lipid peroxidation can be altered under biotic stress and is measured by production of malondialdehyde (MDA) which is a biomarker of oxidative stress (Ibrahim and Jaafar, 2012). Malondialdehyde is produced upon breakdown of membrane lipids and is an indicator of cellular damage caused by pathogen infection (Zeeshan *et al*., 2014). Membrane damage by peroxidation of fatty acids can be initiated by ROS (Gobel *et al*., 2003). These lipids can react with hydroxyl radicals and singlet oxygen to form lipid peroxy radicals and hydroperoxide which enhance membrane lipids peroxidation which in turn causes breakdown of lipid structure and function (Ayala *et al*., 2014). These parameters i.e. lipid peroxidation, protein oxidation and ROS accumulation are commonly used to indicate the level of cell damage caused during various stress conditions (Hakmaoui *et al.*, 2012).

In the present study, the levels of MDA increased significantly in the inoculated plants of susceptible cultivars than in the inoculated plants of tolerant cultivars at 8 wpi and at 12 wpi. This is in line with the findings by Debona *et al*. (2012) who reported that after infecting wheat with the fungus *Pyricularia oryzae*, the levels of MDA increased. Susceptible pepper lines showed increased MDA malondialdehyde content upon inoculation with *Cucumber mosaic virus*, whereas the resistant pepper lines did not show substantial changes in MDA content upon inoculation with the virus (Petrova *et al*., 2012). Contradictory results indicated an increase in MDA in resistant pepper upon infection with *Tobacco mosaic virus* (Madhusudhan *et al*., 2009).

The susceptible cassava cultivars to CBSV had the highest levels of lipid peroxidation compared to the tolerant cultivars thus explaining the severity in cellular damage in the susceptible cultivars than in the tolerant cultivars. Hydrogen peroxide in very high amounts leads to cellular damage expressed as lipid peroxidation. Cultivars resistant to CBSD had lower amounts of H_2O_2 hence they experienced lesser cellular damage. Consequently, they had lower amounts of MDA compared to the susceptible cultivars that had higher amounts of H_2O_2 and lipid peroxidation. Zeeshan *et al*. (2014) suggested that induction of antioxidant enzyme may suppress the level of lipid peroxidation in cells. In this study, resistant cultivars showed increased levels of antioxidant enzymes i.e., POD and APX which probably suppressed lipid peroxidation thus reducing the amount of MDA.

5.5 Antioxidant enzyme response of cassava upon inoculation with cassava brown streak virus

The enzymes catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) are implicated in scavenging H_2O_2 by breaking it into water and oxygen. In particular, CAT and APX are distinguished antioxidant enzymes that detoxify stress-provoked H_2O_2. The two enzymes are meant to down-regulate the impacts of such stress-provoked H_2O_2 to levels that can support normal plant growth and development and defense against abiotic and biotic stresses. Catalase mainly occurs in peroxisomes, whereas APX occurs majorly in chloroplasts, cytosol, mitochondria and peroxisomes. Although APX efficiently degrades even very low levels of H_2O_2, CAT in contrast mainly degrades H_2O_2 in relatively high concentrations. Catalase plays a vital role in both plant metabolism and defense and in signal transduction (Nie *et al*., 2015). Ascorbate peroxidase plays a major role in the ascorbate-glutathione cycle which is a major

H_2O_2 detoxifying system and it detoxifies H_2O_2 into H_2O (Caverzan *et al*., 2012). Ascorbate peroxidase mainly responds with an aim of protecting plants against adverse environmental stress and the activity increases together with that of other enzymes such as CAT and POD.

The amount of ascorbate peroxidase (APX) increased significantly at 8 wpi in all the plants but comparison between all inoculated and non-inoculated plants showed no significant increase in amount of APX. At 12 wpi, the APX activity increased remarkably in the inoculated plants of tolerant cultivars Karibuni, Karembo, and Tajirika but there was no significant increase in the amount of APX in inoculated plants of susceptible plants. The increase in activity of APX could have probably been due to the rapid antioxidant reaction elicited by the optimal levels of hydrogen peroxide. Similar findings were reported by Hernandez *et al*. (2006) whereby upon infection of apricot with plum pox virus, the APX increased in the resistant plants compared to the susceptible plants. In Fujiwara *et al*. (2016) findings, the level of ascorbate which is a substrate for APX increased upon inoculation of resistant *Brassica rapa* plants with Turnip mosaic virus while in the inoculated susceptible plants, the amount of ascorbate declined.

At 8 wpi, there were no significant changes in the activity of CAT in the inoculated plants compared to the non-inoculated plants for all the cultivars. Non-significant changes in amount of CAT were reported in tobacco infected by tobacco mosaic virus (Fodor *et al*., 1997). In the reports by Hakmaoui *et al*. (2012) and Debona *et al*. (2012) after infecting tobacco with pepper mild mottle virus and wheat with *Pyricularia oryzae* respectively, the levels of CAT declined leading to increased levels of foliar H_2O_2. At 12 wpi, the amount of CAT was significantly higher in the plants of all inoculated susceptible and tolerant cultivars than their controls. This

increase in the activity of CAT at 12 wpi could have led to the decline in the levels of H_2O_2 in the susceptible cultivars.

Peroxidase enzyme plays two roles during infection i.e., scavenging high levels of H_2O_2 and enhancing instant defense response against pathogen infection (Sulman *et al*., 2001). Peroxidases are a member of a large multigenic family that is responsible for a number of physiological processes during the life process of a plant (Almagro *et al*., 2008). This participation in the physiological processes is thought to be due to the large range of enzymatic isoforms in PODs and also because of the versatility of their enzyme-catalyzed reactions. Peroxidases are involved in lignin formation in plants, cross-linking of cell wall components and ROS metabolism. The cross-linking creates a physical barrier to inhibit pathogen colonization (Almagro *et al.,* 2008). Peroxidases have also been shown to synthesis secondary metabolites with recognized medicinal properties (Barceló and Pomar, 2002).

In this study, at 8 wpi and 12 wpi, there was a significant ($P \leq 0.05$) increase in the amount of POD in inoculated plants of the tolerant cultivars, whereas the inoculated plants of susceptible cultivars did not exhibit significant increments in the amount of POD at both times. Just like APX, the activity of POD could have been triggered by the peak levels of H_2O_2 in tolerant cultivars. Similar findings were reported by Zeeshan *et al*. (2014) whereby after infecting cotton with cotton leaf curl Burewala virus, the activity of POD increased in the resistant cotton cultivars than in the susceptible cultivars. Similarly, in Riedle-Bauer, (2000) findings, plants of *Cucumis sativus* and *Cucurbita pepo* cultivars resistant to *Cucumber mosaic virus* and *Zucchini yellow mosaic virus* respectively showed higher POD activities than in the plants of susceptible cultivars. Tomatoes resistant to *Tomato yellow leaf curl virus* (TYLCV) showed an increase in

POD activity upon infection with the virus as compared to the susceptible tomato cultivars (Dieng *et al*., 2011). In Madhusudhan *et al*. (2009) report, the increase in the amount of POD in the resistant tobacco and bell pepper upon infection with tobamovirus was higher than in inoculated susceptible tomato and bell pepper.

5.6 Conclusion

Cultivars Karibuni, Tajirika and Karembo were identified as tolerant to cassava brown streak disease in the present study. Cultivars Ebwanatereka 1, Ebwanatereka 2, MM96/7151, Kibandameno, Ex-ndolo, KME-I, Ex-Mariakani, TME 14, TME 204 and TME 7 were identified as susceptible to cassava brown streak disease.

Susceptible cultivars had high content of hydrogen peroxide, whereas tolerant cultivars had reduced amounts of hydrogen peroxide which probably triggered defense mechanisms against CBSD. Infection of cassava by CBSV caused reduction in chlorophyll in susceptible cultivars but the tolerant exhibited little or no chlorophyll reduction.

Inoculation of cassava with CBSV triggered an increase in the activities of peroxidase and ascorbate peroxidase enzymes in tolerant cultivars. Peroxidase and ascorbate peroxidase enzymes therefore could play a vital role in resistance of cassava to CBSD.

The increase in catalase enzyme upon infection of cassava with CBSV in this study was uniform in all cultivars irrespective of the cultivar being tolerant or susceptible. Catalase enzyme therefore might not play a significant role in defense of cassava to CBSD.

5.7 Recommendations

From this study, the following recommendations are made:

i. Peroxidase and ascorbate peroxidase genes in tolerant and susceptible cultivars should be analyzed further by gene expression to assess whether the genes are up regulated in tolerant cassava cultivars during CBSD infection.

ii. Plant breeders should adopt peroxidase and ascorbate peroxidase enzymes in selection of CBSD tolerant cassava cultivars in their breeding strategy. The genes from tolerant cultivars can be incorporated into the susceptible cultivars to induce resistance. Since peroxidase can synthesize bioactive substances such as anti-fungal and anti-bacterial compounds, peroxidase can also be targeted for anti-viral properties against CBSV.

iii. More farmer-preferred cultivars of cassava should be screened for resistance and their antioxidant response compared between resistant and susceptible cultivars to assess whether peroxidase and ascorbate peroxidase will be implicated in resistance as observed in this study.

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