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Exploitation of Natural Resistance Genes, Mutation and Phytosanitation to Eliminate Cassava Geminiviruses

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Exploitation of Natural Resistance Genes, Mutation and Phytosanitation to Eliminate Cassava Geminiviruses

by

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(2426042)

'A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.'

Molecular and Cell Biology

in the Faculty of Science, University of the Witwatersrand, Johannesburg, South Africa

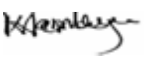
Supervisor: Prof. Chrissie Rey

Co-supervisor: Prof. Evans Nyaboga

September 2024

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

_____9th_____day of__September__2024_____at 12PM (EAT)_____

DEDICATION

To my loving son Leo Mwangi. You made this journey worthwhile.

ACKNOWLEDGEMENT

First, I am grateful to the Almighty God for granting me the strength and good health throughout the study.

I am gratefully indebted to my supervisors Prof. Chrissie Rey and Prof. Evans Nyaboga for their support, continued guidance and wise counsel during this study.

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LIST OF ABBREVIATIONS

ACMV	African cassava mosaic virus
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
bp	Base pairs
CAT	Catalase
CG	Candidate genes
CMD	Cassava mosaic disease
CMG	Cassava mosaic geminiviruses
Ct	Cycle threshold
DAS	Double antibody sandwich
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EACMV	East African cassava mosaic virus
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMS	Ethyl methyl sulphonate
FW	Fresh weight
HR	Hypersensitive response
HSD	Honestly significant differences
HW	Hot water
ICMV	Indian cassava mosaic virus
IITA	International Institute of Tropical Agriculture
JA	Jasmonic acid
KALRO	Kenya Agriculture and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
LSD	Least significant difference
MAS	Marker assisted selection
MDA	Malondialdehyde
NBS-LRR	Nucleotide binding site leucine rich repeats

NLR	Nucleotide-binding and leucine-rich repeat
nm	Nano meter
PCR	Polymerase chain reaction
POD	Peroxidase
PVP	Polyvinyl pyrrolidone
qPCR	Quantitative polymerase chain reaction
QTL	Qualitative trait loci
R	Resistance
RGA	Resistance gene analogs
ROS	Reactive oxygen species
rpm	Revolution per minute
RuBisCO	Ribulose 1-5 biphosphate carboxylase oxygenase
SA	Salicylic acid
SACMV	South African cassava mosaic virus
SAR	Systemic acquired resistance
siRNA	Small interfering RNAs
SNP	Single nucleotides polymorphism
SSA	sub-Saharan Africa
SYBR	Synergy Brands
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TBA	Thio barbituric acid
TBE	Tris boric EDTA
TCA	Trichloroacetic acid
TME	The *Manihot esculenta*
TMS	The *Manihot* species
TYLCV	Tomato yellow leaf curl virus
UV	Ultra violet

CHAPTER ONE

1.0 INTRODUCTION

1.1 Cassava crop and the economic importance

Cassava (*Manihot esculenta* Crantz) is a drought resistant herbaceous plant that is primarily grown for its high starch content (Lukuyu *et al*., 2014). Its ability to grow in poor soils and tolerance to irregular rainfall make it an important crop for food security (Hillocks, 2000). Farmers in tropical regions of the world with limited resources majorly cultivate the crop (Mdenye *et al*., 2016). Because cassava can be cultivated year-round, it can be used as a substitute food in situations where other resources are scarce because of protracted droughts brought on by climate change.

Worldwide, roughly 19 million hectares of land are used for cassava cultivation, yielding an annual production of about 314.8 million tons (FAOSTAT, 2022). Africa, South America, and Southeast Asia are the three main producers of cassava worldwide, with Africa accounting for more than 64.7% of the crop production (FAOSTAT, 2022). With 800 million people relying on it for food globally, cassava ranks fourth in importance among food crops, behind maize, wheat, and rice (Rey and Vanderschuren, 2017; Tafesse *et al*., 2021). The Portuguese introduced the crop to Africa in the sixteenth century, and by the mid-1800s, it was widely grown throughout the continent, serving as a staple crop, particularly in West African nations (Liu *et al*., 2014). Nigeria accounts for almost 20 percent of the world's cassava production and is the world's greatest producer, yielding over 57 million tons of tuberous cassava roots (FAOSTAT, 2022).

According to Akinnagbe (2010) and Fakir *et al*. (2012), the dry matter weight of cassava roots ranges from 30 to 40%, of which is composed of 25.7 to 30% starch, 70% moisture, 2% fiber, 1% proteins, and other materials including minerals. According to Montagnagnac *et al*. (2009),

cassava leaves are also widely eaten as vegetables due to their high protein, iron, zinc, calcium, and potassium content, as well as their high vitamin and mineral content. The most widely used product of the crop is cassava flour, which is made from both fermented and unfermented cassava. Other uses for the crop include sedimented starches, cooked or roasted cassava granules, medicine, drinks (made with cassava components), and cooked fresh roots and leaves as vegetables (Hillocks *et al*., 2002). Cassava leaves and roots are utilized as feed for animals; the leaves offer an excellent supply of proteins, minerals, and vitamins, while the roots are a good source of energy. Consequently, producers use the crop as a cash crop in addition to a source of subsistence.

1.2 Constraints to cassava production

Numerous biotic and abiotic constraints affect the cassava crop, limiting its yield. A few of the abiotic factors that restrict the production of cassava are drought, low soil fertility, root deterioration during storage, lack of improved and disease-free planting varieties, and ineffective farming methods (Devi *et al*., 2022: Alonso Chavez *et al*., 2021). The greatest abiotic threat to cassava production that results in financial losses is disease and pest infestation (Alonso *et al*., 2022; Ebewore and Isiorhovoja, 2019; Bisimwa *et al*., 2019). Cassava brown streak disease, cassava mosaic disease, cassava bacterial blight, and cassava anthracnose disease are the primary diseases, whereas the main pests are cassava mealy bugs, cassava green mites, and cassava grasshopper (Samura *et al*., 2021; Lin *et al*., 2019).

1. 3 Cassava mosaic disease

According to Thresh and Cooter (2005) and Mohammed *et al*. (2012), one of the major constraints to cassava production in sub-Saharan Africa is cassava mosaic disease (CMD), which is brought on by cassava mosaic geminiviruses (CMGs). In extreme cases, yield losses might reach 100%.

Eleven species of cassava mosaic geminiviruses (CMGs) (genus Begomovirus, family Geminiviridae) are responsible for the disease. Nine of these species are found in Africa, two in India and Southeast Asia, and two in the Indian subcontinent and Southeast Asia (ICTV, 2019; Minato *et al*., 2019). Fauquet *et al*. (2005) and Soro *et al*. (2021) report that the virus is disseminated by the usage of infected cuttings and by the whitefly, *Bemisia tabaci*. Fauquet and Fargette (1990) reported that the disease can result in a yield loss ranging from 20 to 95 percent in susceptible cultivars.

1.3.1. Symptoms of cassava mosaic disease

According to Uke *et al*. (2022) and Alabi *et al*. (2011), farmers may readily identify the symptoms of cassava mosaic disease. Leaf chlorosis, stunting, decrease, and deformation, as well as mosaic patterns on the leaves, are all symptoms of the disease (Figure 1). It can be yellow or green mosaic, with the yellow mosaic being more noticeable (Thresh and Cooter, 2005).

These physiological changes affect the development of storage roots resulting in severe size reduction of the storage roots. The severely infected plants do not produce any seed or yield for further multiplication. Even within the same cassava variety, there can be significant differences in the symptoms between shoots, leaves, and plants (Houngue *et al*., 2019). Numerous factors, including various virus strains, plant age, host sensitivity, and environmental factors like soil quality and water availability, might affect the variation in disease severity (Hillocks and Thresh, 2000).

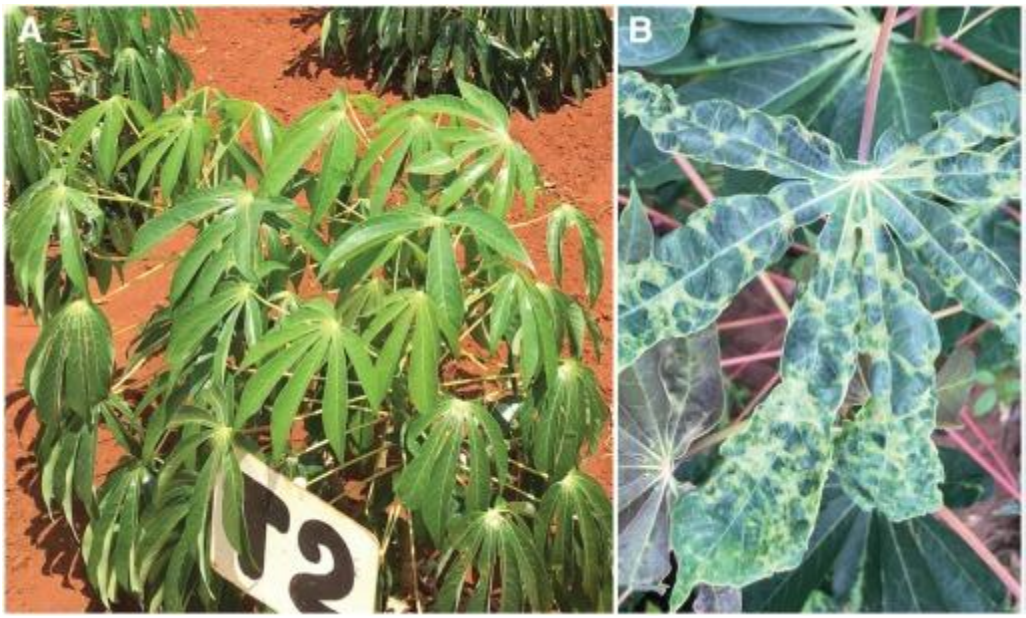


Figure 1.1 Symptoms of cassava mosaic disease on cassava plants in the field.

(**a**) healthy cassava plant in the field; and (**b**) CMD-infected cassava plant showing leaf chlorosis and deformation (Lin *et al*., 2019).

1.3.2 Diagnosis of cassava mosaic disease

There are several traditional and molecular diagnostic tools available for detection of cassava mosaic geminiviruses in cassava plants. Some of the methods used in the detection of cassava mosaic geminiviruses include enzyme-linked immunosorbent assay (ELISA), PCR, quantitative PCR and use of indicator plants.

The diagnostic technique ELISA Abd El-Aziz. (2019) was first utilized to produce the first CMG distribution map for Africa (Swanson and Harrison, 1994), then later extended to provide a more detailed map of the distribution of ACMV and EACMV in East and Southern Africa (Ogbe *et al*., 1997). It is also useful for differentiating between EACMV and ACMV. However, the DNA-based PCR is widely utilized since it has been demonstrated to be more reliable in identifying CMD due to CMG recombination (Legg *et al*., 2001).

Primers specific to the CMD gene are used in the PCR approach to extend complementary strands of DNA. After the initial PCR was discovered, other forms of PCR were created. One such molecular-based method for determining the amount of viral DNA in cassava is quantitative polymerase chain reaction (qPCR). According to Shirima *et al*. (2017) and Lu *et al*. (2012), the technique has high sensitivity in identifying changes in gene expression that arise from diverse sources such as developmental and environmental variations. Although resistant varieties do not exhibit viral symptoms, the method is effective in detecting extremely low viral loads in the varieties (Maruthi *et al*. 2014; IITA, 2009).

Before molecular and serological based techniques were well studied, the use of indicator plants for CMG detection was practiced in the past. This technique uses *Nicotiana benthamiana* as an indicator plant by injecting it with suspected CMD-infected leaf sap thus causing the characteristic symptoms. This confirms the presence of CMD in the suspected plants and it is still a valuable tool for identifying CMD in cassava (Oke and Hussain 1990).

1.3.3 Management of cassava mosaic disease

Numerous strategies have been developed to eradicate the disease, such as rouging symptomatic plants, using planting materials free of disease, thermotherapy, chemotherapy, mutation, and using resistant varieties (Hareesh *et al*., 2023; Uke *et al*., 2021; Uzokwe *et al*., 2016: Rabbi *et al*., 2014). The first two methods are time-consuming and require continuous monitoring to look for signs of disease outbreaks in cassava fields. When combined with tissue culture, chemotherapy and thermotherapy are efficient in getting rid of viruses. Tissue culture is effective in elimination of viruses from planting materials but it is labour intensive, time consuming and is not applicable for use by farmers due to the huge cost required in the establishment of tissue culture facilities.

The best way to control the disease, however, is to utilize resistant cassava varieties (Wagaba *et al*., 2013; Akano *et al*., 2002). Particularly in cultivars that outpace viral buildup, the method reduces the viral burden in the farming system and decreases yield losses from infected plants (Rabbi *et al*., 2014; Houngue *et al*., 2019). Three CMD resistance quantitative trait loci (QTLs); CMD1 (recessive gene), CMD2 (major dominant gene), and CMD3 (QTL conferring resistance) have been found and are used in breeding efforts. Additionally, notable molecular markers linked to CMD2 and CMD3 have been found (Okogbenin *et al*., 2012; Akano *et al*., 2002; Fregene *et al*., 2001). Different cassava cultivars in Africa have acquired the CMD resistance present in Tropical Manihot species (TMS) through the process of molecular breeding (Jha *et al*., 2020).

1.4 Resistance genes and effector triggered immunity

Plants have evolved a defense system against pathogen invasion. Plants go through a series of reactions in response to pathogen attacks in order to prevent infection. A mechanism that is triggered by pathogen attack in plants is the production of pathogen related proteins (Dos Santos and Franco 2023; Kaur *et al*., 2022). Plant receptor molecules identify pathogen-related proteins and trigger defense signaling pathways in the host plant (Abdul Malik *et al*., 2020). When a pathogen invades a plant, resistance proteins (R proteins) trigger a variety of signaling reactions that result in host interactions that are incompatible.

R proteins can be divided into two categories: those that encode the NB-LRR/NLR domain Liu *et al*. (2024); Wu *et al*. (2014) and those that do not. The NLR class, the largest class of R genes in plants, is composed of two highly conserved domains, a central nucleotide binding site (NBS) and a C-terminal series of leucine-rich repeats (Vossen *et al*., 2013; Zhang *et al*., 2014). As per the findings of Jia *et al*. (2013) and Van der Linden *et al*. (2014), the avirulence gene product

recognition is mostly influenced by the LRR domain, whereas the NBS region is presumed to be important for ATP binding and the overall activity of the R gene.

NLR proteins are classified into two according to their N terminal domain: NLRs (TNLs) including coiled coil (CC) containing NLRs and NLRs (TNLs) having toll/interleukin-1 receptors (TIRs) (Bentham *et al*., 2018). Due to the absence of consistent motifs and sequence similarity in the CC domain, CNLs have been classified using motifs in the NB domain rather than the CC domain, which has made it challenging to analyze CNLs using resources like Pfam (Meyers *et al*., 1999; Meyers *et al*., 2002; Meyers *et al*., 2003; Finn *et al*., 2016).

Recent studies on the CC domain have revealed three key characteristics commonly used to characterize its function: (i) the ability to induce cell death upon expression in plant models such as *Nicotiana benthamiana*; (ii) the necessity for self-association to signal cell death; and (iii) the presence or absence of CC-specific motifs such as the EDVID motif. These findings have resulted in the widespread classification of CC domains into several classes, including the solanaceous plant-specific I2-like and SD-CC classes, CCEDVID, CCR, and CCCAN (canonical or classical CC domain). (Hamel *et al*., 2016; Collier *et al*., 2011).

The CCEDVID class, which includes NLRs like Sr33, MLA10, Rx, SlNRC4, Rp1-D21, and RGA5, is named after the highly conserved EDVID motif, which is thought to be involved in intramolecular interactions with the NB domain (Rairdan *et al*., 2008; Bai *et al*., 2012; Wang *et al*., 2015; Leibman-Markus *et al*., 2018). Classical/canonical CCCAN domains include RPS2 and RPS5, as well as additional NLRs that do not fall into any of the first two categories. NLRs with a CC domain resembling RPW8 are classified as belonging to the CCR subclass (Collier *et al*., 2011). The two most common signaling proteins are TIR and CC domains, although their

structures and roles are very different. While CC proteins are mostly helical, crystal structures show that TIR domains have a consistent flavodoxin-like form consisting of five α-helices encircling a five-strand β-sheet (Ve *et al*., 2015).

The phosphate-binding domain (P-loop) and kinase 2 motifs are highly conserved in the NBS region of known R genes and R gene analogs (RGAs). Amplification of sequences between two motifs has frequently been required for cloning NBS sections of RGAs from various species (Deng *et al*., 2000; Noir *et al*., 2001). By cloning and mapping new RGA sequences, it is possible to identify genomic areas that are likely to govern resistance or even candidate R genes, as these sequences have been mapped and proven to be genetically related to known R genes (Meyers *et al*., 1999; Allie *et al*., 2014). Van der Linden *et al*. (2014) stated that fragment cloning, polymorphism discovery, and polymorphism conversion into markers for population separation were the main requirements for mapping the amplified RGA regions.

1.5 Phytosanitation of cassava based on thermotherapy, chemotherapy and phytohormones treatments to obtain a clean seed.

Phytosanitation is a key component for the control of CMD, but the method is rarely used in cassava fields. Physical elimination of CMGs has been practiced through various means including thermotherapy, chemotherapy and phytohormones treatments.

1.5.1 Thermotherapy

Cassava geminiviruses are primarily removed from tissue-cultured plants using the thermotherapy approach, which involves heating the plants inside a thermochamber. Viral replication in plants is inhibited by high temperatures, which also impedes plant-virus contact (Qu *et al*., 2005). Plants exposed to high temperatures decrease the synthesis of viral nucleic acids, which in turn reduces

the migration of the virus into the apical meristem (Wang *et al*., 2008). It has been shown that a combination of thermotherapy and meristem tissue culture can eradicate cassava mosaic virus from cassava (Mwangangi *et al*., 2014; Kidulile *et al*., 2018). The two investigations did, however, demonstrate that the virus could not be completely eradicated without the use of meristem tissue culture. Although tissue culture is a good way to rid cassava of CMD, farmers cannot use it since it is time-consuming, labor-intensive, requires advanced equipment, and has a poor rate of regeneration (Okori and Nakabonge 2016). It would be preferable if there was a straightforward and practical technique that farmers could employ in the field to eliminate CMGs from cassava stem cuttings intended for planting. In this study, the efficacy of hot water treatment in removing EACMV from cassava cuttings was assessed. Farmers can easily implement a unique, straightforward approach by treating stem cuttings with hot water before using them as planting materials.

It has been observed that using heat treatment to eradicate viruses from vegetatively propagated plants works well. Since the high temperatures in heat therapy prevent viral replication, the environment is not conducive to viral survival (Nangozi *et al*., 2016). The use of hot water treatment to eradicate viruses from plants is less expensive and requires specialized knowledge and advanced lab equipment (Kaiser, 1980). According to a study by Abbas *et al*. (2016), hot water treatment worked to eradicate the potato leaf roll virus from potato tubers after the tubers were heated to 37 ºC for three hours. In Nangozi *et al*. (2016) study, cassava plants infected with CMD and CBSD experienced a 25% and 0% reduction in disease incidence, respectively, after being treated with hot water at 55 ºC. In another study by Ling. (2010), tomato seedlings infected with the pepino mosaic virus showed zero virus detectability after two hours of hot water treatment at 50 ºC. This drop in virus infectivity occurred as treatment time increased. Although the virus was

completely eradicated from the tomato seed, the germination of the seeds was affected at a higher temperature of 55 ºC (Ling, 2010).

1.5.2 Chemotherapy

Many agricultural systems have employed chemicals (chemotherapy) to eradicate viruses from plants (Hu *et al*., 2015: Sastry *et al*., 2014: Panattoni *et al*., 2013). Cassava mosaic geminiviruses have been eradicated in CMD using a variety of herbicides, primarily in tissue culture (Okori and Nakabonge 2016). Chemicals function by blocking the synthesis of nucleic acids, which stops viruses from replicating. Furthermore, certain compounds have the power to make plants resistant to viruses. The first line of defense against a variety of diseases in plants is their systemic acquired resistance (SAR) system, which is complemented by these compounds (Oostendorp *et al*., 2001). Increased viral resistance in plants is facilitated by the SAR. When a plant gets infected with a virus, it develops resistance, which stops the virus from spreading inside the plant. According to Smith *et al*. (2009), Tahmasebi *et al*. (2011), Radwan and Ismail (2019), H_2O_2 and phytohormones activate genes related to pathogenesis, which are important for eliminating pathogens in plants. As a result, they may be important in improving SAR.

1.5.3 Phytohormones

In response to biotic and abiotic stress, plants release phytohormones that strengthen their defenses against infections. Phytohormones applied exogenously to plants have been utilized by researchers to increase plant resistance against infections. Plants release four key hormones in response to pathogens: ethylene, SA, JA, and ABA (abscisic acid) (Alazem and Lin, 2015). Examples of compounds of SA and JA that have been linked to systemic resistance against tobacco mosaic virus are methyl salicylate and methyl jasmonate, respectively. Zhu *et al*. (2014) found that

resistance to tomato mosaic virus was induced by the exogenous application of JA followed by SA on leaves. Furthermore, suppressing production of JA and SA in tobacco increased vulnerability to tobacco mosaic virus, according to the research.

Moreover, plants respond to viral infection by activating antiviral pathways through small interfering RNA and SA (Qi Guang *et al*., 2018). SA accumulation is increased in a number of interactions with resistant plant viruses, leading to both systemic and localized resistance (Jovel *et al*., 2011; Baebler *et al*., 2014). It inhibits the development of coat proteins as well as the proliferation, replication, and movement of viruses (Chivasa *et al*., 1997). Because of this, plants that do not accumulate enough SA are very susceptible to viral infection, which can lead to viral accumulation and systemic viral movement (Huang *et al*., 2005; Ishihara *et al*., 2008; Jovel *et al*., 2011; Baebler *et al*., 2014). The SA-mediated pathway is activated when a pathogen infects a plant, initiating a hypersensitive response.

For instance, SA facilitated the development of a hypersensitive response and systemic resistance in *Arabidopsis* infected with the turnip crinkle virus (Kachroo *et al*., 2000). Additionally, SA stimulates the expression of an RNA-dependent RNA polymerase gene, which produces pathogenesis-related proteins and viral RNA silencing, providing SAR against a range of infections (Incarbone *et al*., 2023: Malichan *et al*., 2023: Qi Guang *et al*., 2018: Smith *et al*., 2009; Tahmasebi *et al*., 2011). Because SA is necessary for signal transduction that leads to SAR, plants lacking SA accumulation are more susceptible to disease (Delaney *et al*., 1994). Furthermore, SA is essential for basal immune responses and resistance (R) gene resistance. It also creates a connection between short interfering RNA antiviral machinery and SA-mediated defense. Additionally, SA stimulates the expression of an RNA-dependent RNA polymerase gene, which

produces pathogenesis-related proteins and viral RNA silencing, providing SAR against a range of infections (Incarbone *et al*., 2023: Malichan *et al*., 2023: Qi Guang *et al*., 2018: Smith *et al*., 2009; Tahmasebi *et al*., 2011). Because SA is necessary for signal transduction that leads to SAR, plants lacking it are more susceptible to disease (Delaney *et al*., 1994). Furthermore, according to Baebler *et al*. (2014) and Hunter *et al*. (2013), SA is essential for mediating R gene resistance, basal immunological responses, and forging a link between SA-mediated defense and the antiviral machinery of small interfering RNA (siRNA).

According to Alazem and Lin (2015), exogenous SA treatment strengthens the resistance of susceptible plants against viral infection. The pretreatment of *N. benthamiana* plants with JA and then SA has been shown most recently by Zhu *et al*. (2014) to enhance systemic resistance to tobacco mosaic virus. These plants are more resistant to the virus when treated with JA or SA. Surprisingly, plants with JA pathway impairment showed greater susceptibility and did not accumulate SA in the phloem or leaves. On the contrary, plants with SA pathway impairment showed increased susceptibility without affecting JA levels (Zhu *et al*., 2014). Although it is unknown how JA controls SA production and resistance in compatible interactions, JA may modify early steps in the SA pathway.

In the study by Lozano-Durán *et al*. (2011), the use of exogenous jasmonate decreased vulnerability of *Arabidopsis* to geminiviruses. Plant defense genes can be effectively induced by exogenous application of JA (Lorenzo and Solano, 2005; Howe and Jander, 2008). According to Wasternack (2007), JA has a critical role in both promoting plant development and strengthening plant defense against microbial pathogen attack and insect damage. When exogenous JA was injected into chili plants at high enough concentrations, the severity and incidence of viral diseases

decreased in the plants for a longer period of time than when the plants were treated with pesticides. As a result, JA gives plants long-term resistance to pathogen and insect attack (Awang *et al*., 2015).

It has been demonstrated in several studies that SA and JA work together to give plants resistance to diseases (Yan *et al*., 2013). After inoculating tobacco, pepper, tomato, and *Arabidopsis* with JA and SA, the plants developed increased resistance against turnip crinkle virus, cucumber mosaic virus, and tobacco mosaic virus (Shang *et al*., 2011). But according to Kachroo *et al*. (2000), *Arabidopsis* developed resistance to the turnip crinkle virus by the separate application of exogenous JA and SA. Therefore, the application of phytohormones to plants can function as a low-cost, environmentally friendly biopesticide for the management of plant diseases, including geminiviruses.

Different defense strategies are employed by plants to fend against microbial attacks. The generation of antimicrobial chemicals, hypersensitivity reaction, and reactive oxygen species (ROS) buildup are a few defense responses. One ROS that has two functions is H_2O_2. Firstly, it inhibits the pathogen by causing localized cell death. Secondly, it produces signals that trigger genes associated with pathogenesis and antioxidants in nearby plant tissues. When pathogens infect plants, H_2O_2 is generated, inducing gene expression and the synthesis of enzymes required for plant defense against viruses (Mejía-Teniente *et al*., 2019; Radwan and Ismail, 2019).

The release of H_2O_2 during the early stages of pathogen infection in plants triggers a series of processes that reinforce the cell wall, including lignification and the creation of papillae. Papillae are essential as a barrier that stops pathogens from entering the plant (Troch *et al*., 2014; Sharma *et al*., 2012; Bednarck *et al*., 2009; Clay *et al*., 2009). Hydrogen peroxide also functions as a signaling molecule in plants at low concentrations and shares many characteristics with

phytohormones (Černý *et al*., 2018). Plant immunity against viruses is provided by SAR which is effective in limiting the spread of viruses in plants. According to Radwan and Ismail (2019), H_2O_2 functions as a signaling molecule that triggers the production of genes relevant to disease, hence inducing SAR. The process of inducing viral resistance in plants is attributed to the induction of SA production by H_2O_2. According to research by Radwan and Ismail (2019), pre-inoculation of watermelon plants with cucumber green mottle virus reduced the virus titre in the plants when H_2O_2 was applied exogenously. Hydrogen peroxide poses as a potential candidate ROS for enhancing resistance of cassava to geminiviruses.

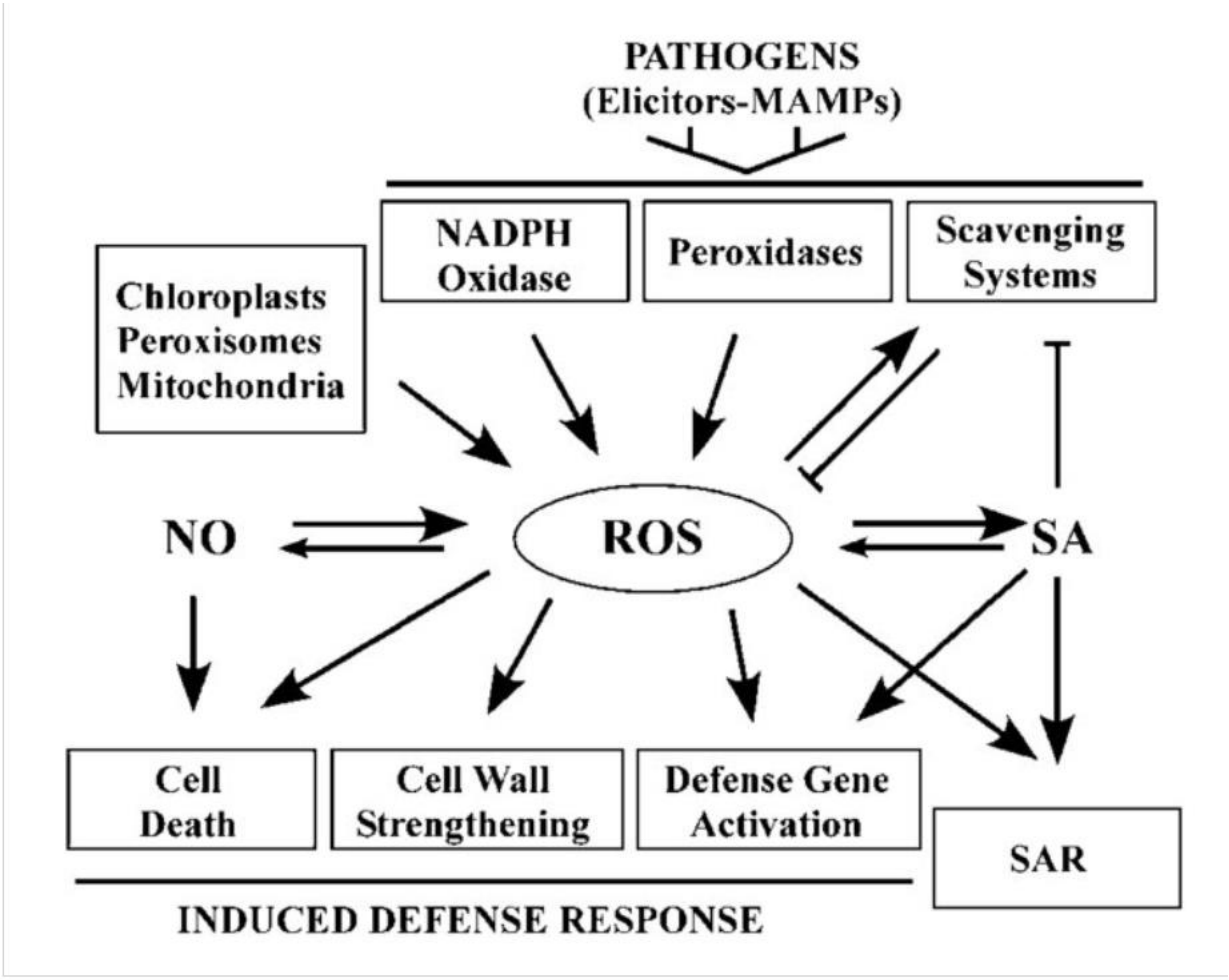


Figure 1.2. ROS production and the role during pathogen attack in plants (Adapted from Torres *et al*., 2006).

1.6. Mutation breeding as a tool to induce virus resistance in plants

Mutagens are physical or chemical agents that alter DNA or RNA structure. The two most often employed mutagens in plant studies are chemical mutagens and ionizing radiation. According to Çelik and Atak (2017) and El-Sayed *et al*. (2012), ionizing radiation can cause mutation in DNA by breaking a bond and substituting a different nucleotide or deleting it entirely. If an amino acid is removed, this can result in loss of protein function. Point mutations produced by chemical mutagens are less harmful than the extensive rearrangements produced by physical mutagens. They result in mutations that lead to substitutions and amino acid exchanges, which alter proteins structures without impairing their functionality. Since chemical mutagens impart genetic variety into higher plants, which is crucial for agricultural breeding programs, they are preferred over physical mutagens for the goal of increasing desirable plant features (Bhat *et al*., 2005). Additionally, chemical mutagens are more effective in boosting these attributes than physical mutagens. Breeders can benefit from chemically induced mutagenesis as it enables a range of mutations to be produced in a single plant through random modifications (Kozjak and Meglič, 2012). It was the first tool used to produce crops with better qualities, such as plant size, and to create new features, like disease resistance and crop variation (Mba *et al*., 2009). In comparison to traditional breeding, induced mutation speeds up the production of plants with desired features.

The important agronomic traits generated by mutation in plants are genotypic and can be passed through generations (Mullins *et al*., 2021; Oladosu *et al*., 2015) Over the years, improvement of cassava has relied majorly on conventional breeding (Jennings and Iglesias, 2002). However, conventional breeding is quite laborious and time consuming (Shimelis and Laing, 2012).

Chemical induced mutation poses a huge potential to improve cassava and overcome the challenges that come with conventional breeding. Two cassava mutants have been reported so far; mutant Tebankye in Ghana and Fuxuan 01 in China (Maluszynski *et al*., 2000; Yan *et al*., 2013). Tebankye had improved traits of large sized starch granules in the tubers and tolerance to ACMV. Putative mutant lines of cassava with improved yield and starch content were generated through gamma-irradiaton of the cassava stakes (Khumaida *et al*., 2015; Khumaida *et al*., 2017). Therefore, there is a need to exploit the potential of chemical-induced mutagenesis in cassava by generating CMD resistance in farmer-preferred susceptible germplasm.

1.7. Problem statement

Cassava mosaic disease and cassava brown streak viruses are among the most serious threats to cassava production in Africa. In genotypes that are susceptible, CMD can result into yield losses of up to 100% (Akano *et al*., 2002). In Africa, CMD causes up to 440 million USD in economic losses yearly. According to Okogbenin *et al*. (2012), field research has demonstrated that cassava cultivars harboring the CMD1, CMD2, and CMD3 loci exhibit moderate to severe susceptibility to CMD, but they also fully recover from the virus. The genes and underlying molecular mechanism responsible for the three loci contribution to recovery from CMGs, however, remain unclear (Kuria *et al*., 2017). For instance, TME3 and T200 both have positive CMD2 markers, but TME3 is tolerant and T200 is vulnerable, indicating that additional loci may be implicated.

A single area on chromosome 8 is responsible for between 30 and 66% of the genetic resistance found in the African cassava germplasm, according to Wolfe *et al*. (2016). Since CMD 2 is highly heritable and stable and offers long-lasting protection against CMGs, more than 100 million people in Africa rely mostly on it to protect cassava against CMD (Beyene *et al*., 2016; Rabbi *et al*., 2014;

Okogbenin *et al*., 2013). Nevertheless, as Ndunguru *et al*. (2016) have noted, this barrier is weak and is easily broken. Consequently, more research is required to determine which resistance (R) proteins in cassava germplasm cause effector triggered immunity (ETI) which is more specific to the pathogen and is more stable.

This was accomplished in this study by screening cassava germplasm that was either CMD-tolerant or resistant in order to obtain the R gene, whose resulting markers can be used in the future to breed new cassava varieties that are climate change tolerant for sustainable agriculture. In addition, when farmers prefer certain varieties of cassava but the crop is susceptible to CMD, it is imperative to investigate alternative mechanisms of resistance. Therefore, another option is to use mutation induction to induce resistance to CMD. It has not been well utilized to induce mutations in susceptible farmer-preferred cassava cultivars to increase resistance to CMD.

The use of infected planting materials and insect vectors accounts for the continued high rates of viruses and related diseases on cassava in Africa. The risk of cassava geminivirus accumulation rises when new crops are established through vegetative propagation utilizing cuttings from older crops. This makes macro-propagation of appropriate, highly productive, virus-free germplasm necessary. The large-scale *in vitro* multiplication of disease-free cassava planting material is one of the current strategies to reduce cassava viral infections. For small-scale farmers, however, *in vitro* multiplication is not economical and thus unaffordable. Thus, before planting, techniques for sanitizing cassava stem cuttings in the field must be developed.

While many plants have benefited from the use of hot water treatment as a phytosanitary method to eradicate viruses in the fields, cassava has not benefited from this practice as much. The cassava mosaic virus may be eliminated from cassava by using hot water. It might be a straightforward yet

efficient method of ridding cassava of the pathogen. Likewise, the use of phytohormone therapy as a phytosanitary measure to eradicate cassava geminiviruses has not been extensively adopted. Phytohormones can be used to eradicate the cassava mosaic virus because they have the ability to give plants a natural defense against viruses. Furthermore, H_2O_2 functions similarly to phytohormones and can cause systemic acquired resistance in plants. Because hydrogen peroxide could increase the resistance of cassava to geminiviruses, it was used in this study

1.8. Justification of the study

The best way to mitigate cassava mosaic disease (CMD) is to employ resistant cassava cultivars. When combined with less expensive techniques like applying Phytosanitation and phytohormones, these strategies could offer an integrated approach to the management and control of CMD. In addition to being excellent material for the breeding of better cassava varieties, the identified tolerant/resistant varieties can be given to farmers for cultivation. Furthermore, breeding efforts can be accelerated significantly and germplasm can be efficiently characterized at the molecular level using genomic studies. The possibility to employ the identified R genes for CMD resistance in breeding programs or genetic transformation, namely through the application of gene editing technologies, is provided by marker assisted selection (MAS). The identified cassava resistance proteins in this study can be explored in future to obtain resistance gene markers that will speed up conventional breeding programs.

Further to this, since cassava varieties resistant to cassava mosaic disease are available, their genes can be explored to induce resistance in susceptible varieties thus enhancing the yields of cassava. Therefore, it is necessary to develop new varieties that are adapted to CMD by applying marker assisted selection (MAS) or genetic transformations based on useful candidate genes.

Because mutation induction is inexpensive, it is still a valuable complimentary tool in cassava breeding programs. Gamma irradiation provides a well-established method of inducing mutations in cassava. The irradiation method was utilized in the early 1990s to create the *Tek Bankye* cassava mutant (Asare and Safo 1997). Several other mutant plants with enhanced disease resistance have been generated in prior investigations. Chemical mutagens offer a low-cost, easily scalable means of introducing resistance in plants but their application in cassava cultivation has not been well established. Cassava is a strong candidate for mutation induction for CMD resistance in susceptible yet farmer-preferred cultivars. Chemical mutagen ethyl methyl sulfonate (EMS) was used in this study to induce mutation in CMD susceptible cassava varieties. This will provide a low-cost method to eradicate disease in susceptible varieties and significantly alleviate food insecurity.

Additionally, it is necessary to create simple, affordable techniques that farmers may easily implement to lower the viral loads in planting material especially when the starting material is virus infected. Hydrogen peroxide and phytohormones have been extensively used to induce plants resistance against viruses and other diseases. Applying them exogenously may provide a low-cost, straightforward, and efficient way to get rid of cassava geminiviruses from infected stem cuttings. Given that hot water treatment has been demonstrated to be effective in eliminating viruses from plants, it may be a simple yet very successful method of eliminating geminiviruses from cassava.

1.9. Aims and Objectives

There were two aims of this study:

Aim 1 To explore natural resistance to cassava mosaic disease (CMD) in selected cassava germplasm in order to use this information in developing new resistance markers in the future

Objective (i) To screen cassava germplasm for CMD resistance.

Objective (ii) To identify virus resistance genes from other host plants and obtain their gene homologs in cassava and design primers from the homologs.

Objective (iii) To determine their level of expression of R genes in resistant, susceptible and tolerant cassava varieties at various times after inoculation using the designed primers.

Aim 2 Since many national institutes in African nations lack the necessary funding to apply genetically based CMD prevention strategies, it would be ideal to have alternative strategies that would be easily adopted by small-scale rural farmers.

Objective (i) To cause mutations in cassava that confer genetic resistance against EACMV.

Objective (ii) To ascertain whether treatments with hot water, hydrogen peroxide, and phytohormones are effective in eliminating EACMV.

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CHAPTER TWO

2.0 IDENTIFICATION AND CHARACTERIZATION OF NBS-LRR GENES IN CASSAVA AND THEIR EXPRESSION PROFILES IN SUSCEPTIBLE AND RESISTANT CASSAVA VARIETIES IN RESPONSE TO EAST AFRICAN CASSAVA MOSAIC VIRUS INFECTION

Abstract

The use of host CMD-resistant genes and cultivation of resistant cultivars are the two most economical and successful ways to manage CMD. Nucleotide-binding site-leucine-rich repeat (NBS-LRR) generating genes make up the bulk of disease resistance (R) genes in plants and are involved in defense against a range of diseases. In this work, seven NBS-LRR members were identified from the cassava genome through bioinformatics, and their gene and protein properties (phylogenies, physicochemical properties, gene structures, and conserved motifs) as well as their expression patterns in response to geminivirus infection were examined. The expression patterns of the selected MeNBS-LRR genes were analyzed using RT-qPCR in the leaves of two resistant (Karembo and Tajirika), one tolerant (Migyera) and two susceptible (Mucericeri and Ex-Ndolo) cassava varieties after infection with EACMV at 12, 32 and 67 dpi. The physicochemical properties of the selected MeNBS-LRR proteins showed significant variations in the protein lengths, molecular weights and isoelectric point. Every gene had one to six exons. A total of fifteen conserved motifs with amino acid residues varying from 9 to 50 were also found. The expression of the seven MeNBS-LRR genes varied over time between the susceptible and resistant cassava varieties. MeNBS-LRR gene expression patterns revealed that they function during various stages of EACMV infection. At 12, 32, and 67 dpi following EACMV infection, the expression of every NBS-LRR gene was considerably higher in the resistant cassava varieties than in the susceptible cassava. The susceptible cassava cultivars showed a general tendency of reduced gene expression. MeNBS-LRR genes have been implicated in the induction of CMD resistance in cassava, as evidenced by the discovery and expression studies of these genes.

2.1 Introduction

Plants use a first line of defense called pathogen-associated molecular patterns (PAMPs) to recognize pathogens. According to Jones and Dangl (2006) and Chen and Ronald (2011), PAMP-triggered immunity (PTI) is the first line of defense that eliminates a large number of pathogens while the other systems impede infection. During the infection, there is production of avirulence proteins (Avr), one of their means of suppressing host defenses and inducing infection, that are recognized by the secondary defense system. According to Nurnberger *et al*. (2004), this is known as effector-triggered immunity (ETI) and is predicated on the ability of disease resistance (R) proteins to recognize particular pathogen effectors. Microbial infections are hindered from spreading by the quick defense response that ETI triggers, known as the hypersensitive response (HR) (Greenberg, 1997). (Zvereva and Pooggin *et al*.,2012). A systemic acquired resistance (SAR) response to such a localized pathogen attack provides prolonged defense against subsequent pathogen attacks (Chen and Ronald, 2011; Cui *et al*., 2015; Abdul Malik *et al*., 2020). In the event of a pathogen invasion, the induction of disease resistance by the R genes is one of the most important plant defensive mechanisms. This leads to a host-pathogen interaction that is incompatible.

The most common class of resistance genes (R genes) in plants is the NBS=LRR, and it is made up of two conserved domains: a C-terminal series of leucine-rich repeats (LRR) and a central nucleotide binding site (NBS) (Vossen *et al*., 2013; Zhang *et al*., 2014). While LRR is the primary determinant in pathogen recognition and protein-protein interactions that establish disease resistance responses, the NBS domain plays a significant role in signaling through the binding and hydrolysis of ATP and GTP (Jia *et al*., 2013; Van der Linden *et al*., 2014). The NBS-LRR containing proteins (NLRs) play important roles in the innate immune system.

Based on their N-terminal domains, NLR proteins are classified into two subclasses: Toll/interleukin-1 receptors (TIRs) containing NLRs referred to TNLs (for TIR-NBS-LRR) and coiled coil (CC) containing NLRs referred to CNLs (for CC-NBS-LRR) (Bentham *et al*., 2018). According to Anderson *et al*. (2018) and Kourelis and Van Der Hoorn (2018), these proteins function as pathogen sensors. When the LRR domain interacts with pathogen effector proteins, the TNL or CNL proteins undergo a conformation change that causes the multimerization of TIR or CC domain and activate signal transduction pathways involved in immune system activation.

The functions of NBS-LRR genes have been studied in a number of plant species. The NBS-LRR gene has been found to have homologs in a wide variety of plant species, including *Arabidopsis* (Meyers *et al*., 2003), *Brassica* (Mun *et al*., 2009), soybean (Kang *et al*., 2012), potato (Lozano *et al*., 2012), cassava (Lozano *et al*., 2015), and others. Many diseases in plant-pathogen interactions, including powdery mildew in sunflower (Neupane *et al*., 2018) and *Vitis vinifera* (Goyal *et al*., 2020), various pathogens in yams (Zhang *et al*., 2020) and cotton (Shi *et al*., 2018), downy mildew and black rot in Chinese cabbage (Liu *et al*., 2021), have been linked to resistance to these genes.

Moreover, R genes that confer virus resistance have been found in tobacco, potato (Tameling and Baulcombe 2007), tomato (Ishibashi *et al*., 2014; Kawamura *et al*., 2014; Lanfermeijer *et al*., 2003), tobacco, soybean (Chowda *et al*., 2011; Wen *et al*., 2011; Khatabi *et al*., 2013; Seo *et al*., 2011; Seo *et al*., 2009), mung bean (Maiti *et al*., 2012) among others.

As a vegetatively propagated crop, cassava is vulnerable to pathogens particularly cassava mosaic geminiviruses, the causal agent of cassava mosaic disease (Thresh and Cooter, 2005). Cassava mosaic geminiviruses can affect the yield of infected cassava crops and can result to 100% yield loss (Torkpo and Amponsah, 2024; Houngue *et al*., 2019). According to Mohammed *et al.* (2012),

one of the greatest obstacles to cassava production in sub-Saharan Africa (SSA) remains cassava mosaic disease (CMD). The most sustainable, environmentally friendly, and farmer-friendly method for managing CMD is to utilize genetic resistance mechanisms based on host-pathogen interactions. Examining the geminivirus-responsive NBS-LRR genes in cassava is crucial given the crucial function that these genes play in the plant defense system against a variety of diseases.

A total of 228 NBS-LRR type genes were discovered on the Phytozome 13 platform of the cassava genome sequence after investigation of NBS-LRR genes in cassava as reported by (Lozano *et al*., 2015). Additionally, four NBS-LRR genes for additional investigation were discovered by Zhang *et al*. (2022) from the transcriptome databases of Lozano *et al*. (2015) and Utsumi *et al*. (2016). They discovered that these genes were stimulated by SA treatment during *Xanthomonas axonopodis* pv. manihotis (Xam) infection. Similarly, NBS-LRR genes were linked to resistance against the cassava anthracnose disease, according to Utsumi *et al*. (2016). Research by Louis and Rey (2015) suggests that several R proteins may be involved in CMD tolerance in the cassava landrace TME3. The findings of the study demonstrated that tolerant TME3 infected with SACMV developed several resistance gene analogs (RGAs) encoding various conserved resistance protein analogs (RPAs). According to the study, TME3 exhibits tolerance to SACMV because of increased expression of 12 SACMV-responsive RGAs, which were not overexpressed in the susceptible T200 landrace. More R genes that may provide resistance against cassava mosaic geminiviruses and be employed in molecular breeding initiatives for CMD resistance need to be investigated.

In the present study, seven R (CC-NBS-LRR) genes previously demonstrated to confer resistance to viruses in other crops Sett *et al.* (2022) were selected and their orthologs retrieved from the National Centre for Biotechnology Information (NCBI) and Phytozome 13 databases. The

homologs of the selected NBS-LRR genes were identified in the cassava genome. The study involved an analysis of the structural diversity, classification based on protein architectures, and phylogenetic relationships of the genes. The expression profiles of these NBS-LRR genes were determined using quantitative real-time PCR (qRT-PCR) at different time points (at early and late stages of virus infection) following EACMV infection of cassava varieties with varying levels of resistance. The screening of cassava R genes was done in this study, which will expedite the breeding of virus-resistant cassava varieties and future studies on the functional characterization of MeNBS-LRRs for disease resistance.

2.2 Materials and methods

2.2.1 Genomic data search, sequence retrieval and identification of NBS-LRR genes in cassava genome

Fourteen resistance (R) genes conferring resistance to viruses in various crops (*Solanum lycopersicum*, *Capsicum annum*, *Capsicum chinense*, *Vigna mungo*, *Glycine max* and model plant *Arabidopsis thaliana*) Sett et al. (2022) were identified (Table 2.1). The gene sequences were retrieved from the NCBI GenBank database. The sequences were aligned to the cassava genome using Geneious software version 2022, used to basic local alignment search (BLAST) for gene orthologs in cassava genome using Geneious software version 2022. The best orthologs were selected from the results based on the e-value (0.05 cut off), conserved domain and percentage homology match. The candidate cassava NBS-LRR sequences were subjected to NCBI-CDD tool for verification of the conserved domains (Yang *et al*., 2020). Seven genes that were described as belonging to the CC-NBS-LRR gene subclass were selected from fourteen genes and used in the current study. The amino acid sequence and gene sequences of the seven orthologs were obtained from the Phytozome 13 database and used for genetic characterization (conserved motifs, gene

structure analysis), phylogenetic analysis and primer design for gene expression studies. The *Manihot esculenta* genomic, protein and coding sequences were downloaded from the Phytozome database (https://phytozome-next.jgi.doe.gov/). The amino acid length, theoretical isoelectric point and molecular weight of the NBS-LRR proteins were predicated using Protoparam tool. The gene ID, chromosomal location, gene and protein sequences information was retrieved from the cassava phytozome 13.

Table 2.1 Resistance (R) genes from other crops and their orthologs in cassava

Resistance gene	Crop plant	NCBI gene ID.	Cassava gene ortholog	Reference(s)
Tm 2	*Solanum lycopersicum*	101255420	Manes.10G102500	Ishibashi *et al*. (2014) Kawamura *et al.* (2014) Lanfermeijer *et al*. (2003)
Pvr 4	*Capsicum annum*	124891889	Manes.11G052160	Kim *et al*. (2017)
Pvr9	*Capsicum annum*	772242483	Manes.09G034700	Kim *et al*. (2015) Tran *et al*. (2015) Janzac *et al*. (2009)
Tsw	*Capsicum chinense*	1068294075	Manes.03G063225	Ronde *et al*. (2013)
CYR1	*Vigna mungo*	936626859	Manes.11G004600	Maiti *et al*. (2012)
RSV3	*Glycine max*	100818461	Manes.03G124100	Chowda *et al*. (2011) Wen *et al*. (2011) Khatabi *et al*. (2013) Seo *et al*. (2011) Seo *et al*. (2009)
HRT	*Arabidopsis thaliana*	834367	Manes.18G105800	Chandra-Shekara *et al*. (2004) Zhao *et al*. (2000)

All seven R genes selected in this study belong to the subclass CC-NBS-LRR. These are coiled coil (CC) containing NLRs referred to CNLs.

2.2.2 Evolutionary history and phylogenetic relationship analysis of the selected NBS-LRR genes in cassava

The evolutionary history and analysis were determined using the Neighbor-Joining method in MEGA 11 software. The bootstrap consensus tree obtained from 1000 replicates was considered to depict the evolutionary history of the analyzed taxa.

2.2.3 Conserved motif and gene structure analysis

The Multiple Expectation Maximization for Motif Elicitation (MEME, version 4.9.1) suite tool (refer to https://meme-suite.org/meme/tools/meme) was employed to ascertain the conserved motifs within specific cassava NBS-LRR genes, as documented by Bailey *et al*. (2015). Parameters were fine-tuned for a motif width ranging from 6 to 50 bp, and a maximum of 15 motifs. The Gene Structure Display Server (GSDS) 2.0 was then used to illustrate the conserved motifs and intron-exon structures of a subset of cassava NBS-LRR genes.

2.2.4 Homology modeling of selected NBS-LRRs in cassava

The chosen NBS-LRR gene in cassava was subjected to homology modeling to predict its 3D protein structure. Using the projected NBS-LRR amino acid sequence, a three-dimensional protein structure was constructed using the SWIISS MODEL software's homology modeling approach (Bordoli *et al*., 2009). The target protein sequence alignment with the template structure served as the basis for this accomplishment. The SWISS-MODEL program makes use of experimentally determined family member structures as templates.

2.2.5 Cassava plant materials, inoculum source and growth conditions

The cassava plant material comprised of eight varieties (Tajirika, Karembo, Migyera, Mucericeri, Fumbachai, Matuja, MH95/0183 and Ex-Ndolo) was obtained from Kenya Agriculture and Livestock Research Organization (KALRO), Food Crops Research Institute, Embu, Kenya. The plants were taken to the Kenya Plant Health Inspectorate Service, Plant Quarantine and Biosafety station, Kenya where the experiment was carried out. Prior to collecting the stem cuttings, the top three leaves from each plant were sampled and placed in plastic bags. These samples were then transported to the laboratory in cooler boxes for testing of EACMV and ACMV using PCR, following the method described by Alabi *et al.* (2008). Stem cuttings from cassava plants that tested negative for ACMV and EACMV using PCR were selected for the subsequent experiments. These stem cuttings were then individually planted in plastic pots (Kenpoly, Kenya), which were filled with a mixture of sterilized soil and sterilized farmyard manure in a ratio of 1:3 (v/v). The pots were irrigated to field capacity once daily until sprouting, and then twice per week thereafter. The sprouted plants were allowed to grow and were kept in a controlled glasshouse with a photoperiod of 16/8 h (day/night), a temperature of 26/16±2 ºC (day/night), and 70% relative humidity, awaiting inoculation with EACMV. The source of inoculum was a CMD-susceptible cassava variety MM96/4884 with a severity score of 5 exhibiting total distortion of 4/5 of the leaves as described by Houngue *et al*. (2019). The plants were collected from farmers' fields and established in a separate greenhouse to serve as a source of EACMV inoculum. The inoculum was confirmed to be positive for EACMV and negative to ACMV through PCR as described by Alabi *et al*. (2008).

2.2.6 Infection of cassava with EACMV, CMD symptom severity scoring and sample collection

The inoculum (the EACMV-infected cassava variety MM96/4884) was grafted onto the test plants to enable the transfer of the viral particles, while the clean test plants of the eight different cassava varieties served as the rootstock (Anjanappa *et al*. 2016). Using a sterile blade, a vertical slit was cut on the stem of clean plants. In order to align the phloem and xylem of the two plants, a scion of the inoculum plants was cut and placed into the slit of the clean test plants. To allow for union, the graft sections were firmly taped using parafilm. To protect grafted plants from excessive evaporation, plastic bags were placed over them and were raised to avoid contact with the plants. After 7 days, the polythene bags were removed. Control plants were mock graft-inoculated with disease-free scions of cassava variety MM96/4884. Each cultivar consisted of five pots (five biological replicates) and repeated three times. All the plants were kept in an insect-proof glasshouse at a temperature of 26/16±2 ºC (day/night) and 70% relative humidity until ready for inoculation with EACMV.

The graft-inoculated plants were monitored weekly for CMD symptoms expression and symptom severity scores were recorded over a period of 70 days. The symptoms were scored on a disease severity scale of 1 to 5, according to Houngue *et al*. (2019). "1 represents no symptoms on the leaves; 2 represents mild chlorotic spots or some base distortion; 3 represents severe chlorotic spots on the entire leaf surface with twisting; 4 represents distorted or shrunken leaf blades (2/3 of the leaf area); and 5 represents severe CMD symptoms and/or total distortion of 4/5 of the leaf area and plant stunting". ANOVA was performed on the severity score data using Genstat 15th edition software, with differences between means separated by least significant differences (LSD)

at $P < 0.05$. Weekly severity score data were recorded for 10 weeks in triplicates and entered into an excel spreadsheet.

Leaves below the apex for EACMV- and mock-inoculated plants were sampled at the three time-points 12 days post-inoculation (dpi), which represent early infection, 32 dpi, which represent early infection and movement and 67 dpi which represents late infection stages (Allie *et al*., 2014). Leaves were collected from five biological replicates for each variety, each treatment (EACMV- and mock-inoculated) and at three time points. Each biological replicate was tested in three independent technical replicates. Leaves from the different time points were sampled, immediately frozen in liquid nitrogen and stored at -80 ºC until subsequent use for DNA and RNA extractions.

2.2.7 Detection of EACMV by conventional PCR

The PCR analysis for EACMV infection was conducted on the collected samples following the method outlined by Alabi *et al.* (2008). The procedure for extracting the whole genomic DNA was performed in accordance with the protocol by Osena *et al*. (2017). A standard thermocycler (Eppendorf AG 22331, Hamburg, Germany) was used to carry out the PCR reaction in a final volume of 25 µl. This volume comprised 12.5 µl of Quick-Load Taq 2× master mix (New England Biolabs), 0.5 µl of 10 µM for both the forward and reverse primers, 2 µl of 50 ng of DNA template, and 9.5 µl of molecular grade water. The PCR thermocycling conditions were as follows: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 60 s and final elongation at 72 °C for 10 min.

2.2.8 RNA isolation and quantification

Total RNA was isolated from leaves below the apex of EACMV- and mock-inoculated plants at time-points of 12-, 32-, and 67-days post-inoculation using CTAB, as described by Chang *et al*. (1993). Following the addition of 0.33 volume of 10 M LiCl and overnight incubation at -20 ºC, RNA was precipitated. Subsequently, centrifugation at 12,000 g for 20 minutes was performed to recover the RNA, and the pellet was resuspended in 250 µl of DEPC water. After a 30-minute incubation at 37 °C with 35 units/ml of RQ1 RNase-free DNase (Promega), the solution was subjected to extraction first using phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and then using chloroform/isoamyl alcohol (24:1 v/v). RNA was precipitated using ethanol, and the resulting pellet was resuspended in 20 µl of DEPC water. The quantity of RNA was assessed spectrophotometrically at 260 nm, and the OD260/OD280 absorption ratio was determined using a NanoDrop 800 UV-vis spectrophotometer (Thermo Fisher Scientific, Inc. USA). The quality and integrity of the RNA were evaluated through gel electrophoresis using 1.5% agarose gel.

2.2.9 Complementary DNA (cDNA) synthesis

The total RNA was used for the synthesis of cDNA using the MMLV II cDNA synthesis kit. The first reaction consisted of 7 µl of molecular grade water, 1 µl each of oligo-DT and random primer and 3 µl of total RNA (1 µg). The mixture was centrifuged and incubated at 70 ºC for 5 min. The second reaction mix was prepared with 4 µl of molecular grade water, 1 µl of dNTPs, 2.5 µl of MMLV buffer and 0.5 µl of MMLVII-RT enzyme. The mixture was added to the first reaction mixture and mixed by vortexing. The resultant cDNA was used for quantitative real-time PCR.

2.2.10 Quantitative real-time PCR (qRT-PCR) assay and qRT-PCR data analysis

Quantitative real-time PCR with cDNA as the template was used for the gene expression studies. Primer3blast software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) was used to build specific primers (Table 2.2) from each of the chosen cassava NBS-LRR genes. With a PCR product size of 95–113 bp, a primer length sequence of 18–25 nucleotides, a Tm of 50–60 ºC, and a GC content of 50–60%, the primer pairs were designed to span exon-exon junctions. To verify amplification of the target region, each primer was examined using gDNA and cDNA samples. Using an ABI 7500 Fast real-time PCR equipment (Applied Biosystems, Foster City, CA, USA), real-time RT-PCR was carried out using the FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany).

In order to detect primer dimers or DNA contamination, three technical duplicates of each biological replication for each cassava variety at each time point were carried out for each NBS-LRR gene in a single plate, together with controls (negative reverse transcription control and no template control). 10 mM of forward and reverse primers and 2 µl of 10-fold diluted cDNA with DNase/RNase free water were added to reactions with a total volume of 20 µl. A dissociation curve was followed by 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C for the PCR. The program LinRegPCR was used to calculate the PCR efficiencies (Ramakers *et al*., 2003). Using qBase software, relative gene expression was computed from the Ct values (Hellemans *et al*., 2007). For mRNA expression, *Manihot esculenta* protein phosphatase 2A (PP2A) served as the reference gene.

Table 2.2 Description of qRT-PCR primers used to determine the selected cassava NBS-LRR gene expressions at transcript level upon EACMV infection.

Gene name	Primer sequence (5′-3′)	Expected amplicon size (bp)
Manes.11G004600	F GGATGGCGTCTCCTGAAGTA R GCACGCCCACAATAGAGTTT	102
Manes.03G063225	F TGAAATAGCCCATCCTGGTC R CAATGCCTCCCAGACTTCAT	112
Manes.18G105800	F TTTCCTGGATGGAGTGAAGG R CATCTTGCCTTGAATCAGCA	95
Manes.10G102500	F GAGCATGTGCCAGAAGTTCA R GCTGGACTGGCCATAATGTT	102
Manes.03G124100	F TGATCACAAGGGTTGCAAAA R CCCACGCTTCATTTTCAGTT	113
Manes.11G052160	F TCGGATGGTAGCAACTCCTT R ACCCCTCCCATTCCATAAAC	110
Manes.09G034700	F TTTCGGATCCAGTTTTGAGG R TTGGATTTCTCCAGGGTCAG	101
Protein phosphatase 2A (PP2A)	F TGCAAGGCTCACACTTTCATC R CTGAGCGTAAAGCAGGGAAG	107

To determine the change in gene expression, the double delta Ct value (2-ΔΔCT) was calculated using the normalized Ct values to the expression of the reference gene (Hellemans *et al*., 2007). With the aid of graphPad Prism 9 software (San Diego, CA, USA), the expression changes for each gene within each cassava variety at 12, 32, and 67 dpi were further examined. There were three technical replicates and three biological replicates employed.

Figure 2.1 presents the entire protocol used for the gene expression pattern experiment.

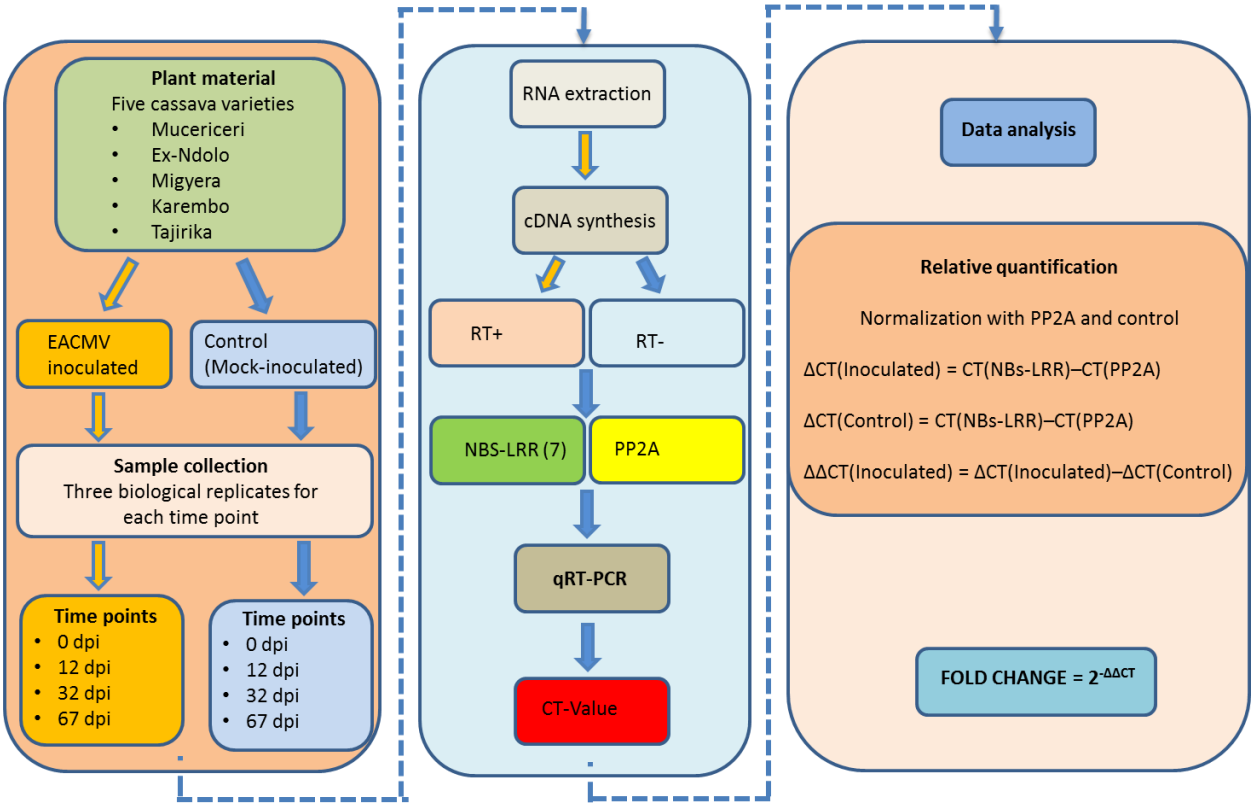


Figure 2.1: Overview of the procedure and data analysis of the expression profiling of selected cassava NBS-LRR genes upon EACMV infection.

2.3 Results

2.3.1 Identification of genes and phylogenetic analysis

Known NBS-LRR genes were retrieved from other plant species (*Solanum lycopersicum*, *Capsicum annum*, *Capsicum chinense*, *Vigna mungo*, *Glycine max* and *Arabidopsis thaliana*) as queries to identify potential genes located on the cassava genome (https://phytozome-next.jgi.doe.gov/), which lead to identification of 14 potential R genes in different chromosomal locations on the cassava genome. The genes were selected from the list of R genes identified in plants as genes that confer resistance to viruses (Sett *et al*., 2022). Among these gene sequences, 7(Manes.11G004600, Manes.03G063225, Manes.18G105800, Manes.10G102500, Manes.03G124100, Manes.11G052160 and Manes.09G034700) annotated as complete genes that carried both NBS and LRR domains and belonging to the subclass coiled coil (CC) containing NLRs referred to as CNLs were selected. The physicochemical characteristics of the 7 CC-NBS-LRR genes in the cassava genome indicated that the length of the CDS ranged from 2700 (Manes.09G034700) to 5163 (Manes.03G063225) (Table 2.3). The lengths of the amino acid sequences ranged from 862 aa (Manes.09G034700) to 1720 aa (Manes.03G063225). The molecular weight ranged from 99.33 (Manes.09G034700) to 196.23 (Manes.03G063225) kDa. The maximum and minimum isoelectric point (pI) of the selected NBS-LRR proteins was recorded in 8.68 (Manes.09G034700) and 5.87 (Manes.03G063225), respectively (Table 2.3). All the selected meNBS-LRR proteins were acidic (pI<7.0) except Manes.10G102500 and Manes.09G034700 which were basic with pI of greater than 7.0 that is 8.56 and 8.68, respectively.

Table 2.3 Characteristics of NBS-LRR genes in cassava.

Gene Locus	Start	End	Chr	CDS (bp)	Pi	MW (kDa)	A. A
Manes.11G004600	548080	552239	Chr11	3236	6.18	122.43	1078
Manes.03G063225	7050354	7063317	Chr03	5163	5.87	196.23	1720
Manes.18G105800	10363519	10369402	Chr18	2796	6.70	107.00	931
Manes.10G102500	25054016	25056824	Chr10	2808	8.56	107.01	935
Manes.03G124100	25538733	25547598	Chr03	3821	6.62	144.89	1273
Manes.11G052160	5617364	5623436	Chr11	4823	6.24	186.09	1632
Manes.09G034700	6732026	6735227	Chr09	2700	8.68	99.33	862

Note: Chr (chromosome); CDS (coding sequence); pI (isoelectric point); MW (molecular weight); A.A (amino acids); kDa (kilo Dalton)

A phylogenetic analysis of the selected NBS-LRR genes revealed their evolutionary history and phylogenetic relationships (Figure 2.2). The NBS-LRR genes were used to identify three primary groups, which were subsequently subdivided into subgroups and clades.

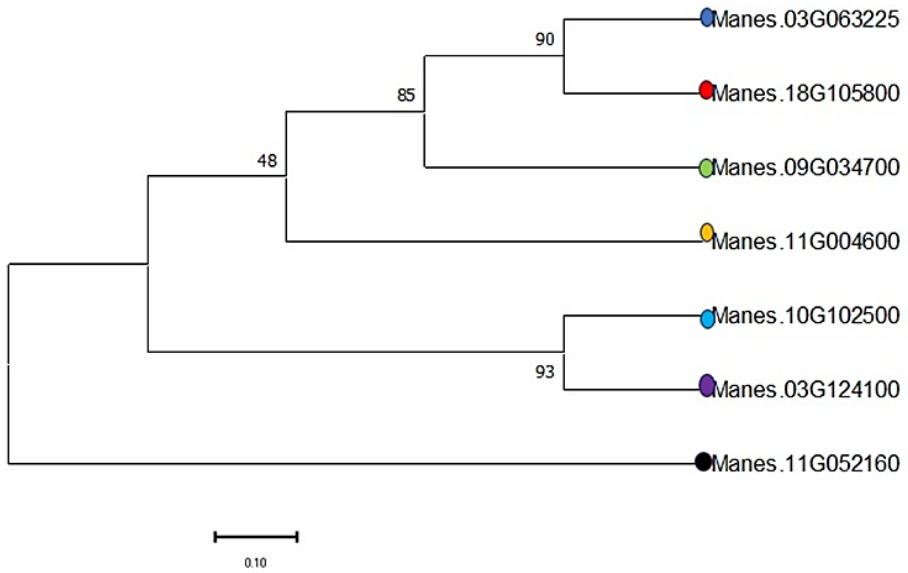


Figure 2.2: Phylogenetic relationship of selected MeNBS-LRRs using the maximum likelihood method

The branches representing partitions replicated in fewer than 50% of bootstrap replicates were collapsed. Next to the branches are the percentages of replicate trees showing that the corresponding taxa clustered together in the bootstrap test (1000 repetitions). The evolutionary distances, measured in base substitutions per site, were computed using the Maximum Composite Likelihood technique. This study involved the examination of seven NBS-LRR nucleotide sequences. Ambiguous positions were removed for every sequence pair (pairwise deletion option). The final dataset comprised a total of 11820 positions. Analysis was carried out using MEGA 11 software.

2.3.2 Gene structure and conserved motif analysis of selected cassava NBS-LRR genes.

Exon-intron arrangements of the genes (Figure 2.3) were created by comparing their coding sequences with the corresponding genomic sequences using the web program GSDS2.0 (http://gsds.cbi.pku.edu.cn/) to better comprehend the gene structures of the seven NBS-encoding R genes. There were one to six exons in each gene. Only one gene, Manes.03G063225, had the greatest number of exons (6) while three genes, Manes.09G034700, Manes.11G004600, and Manes.10G102500, had the least.

There were anywhere between 0 and 6 introns in each gene. Manes.03G063225 and Manes.11G052160 had the greatest number of introns, while three genes (Manes.09G034700, Manes.11G004600, and Manes.10G102500) had none at all.

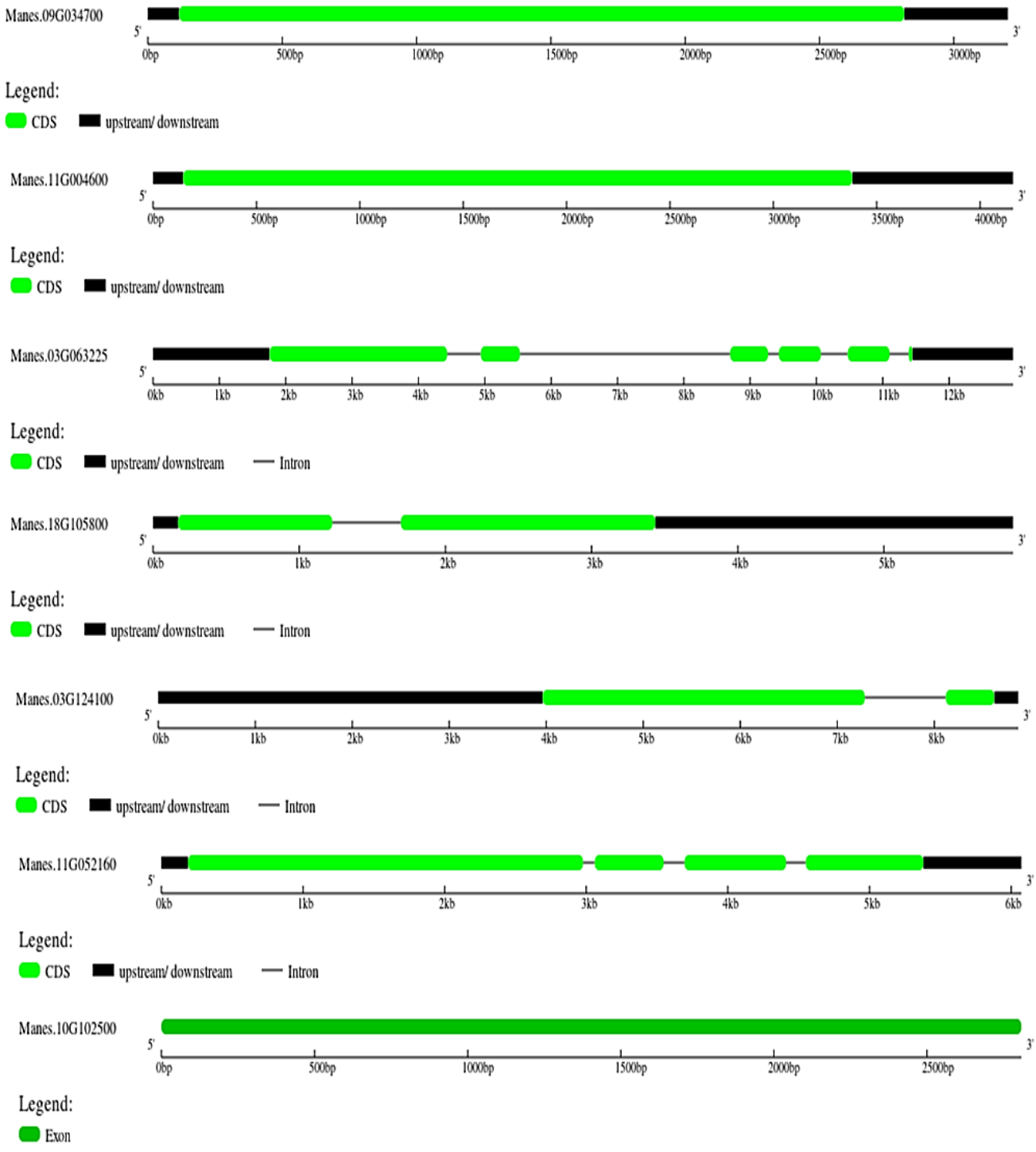


Figure 2.3: Exon-intron structures of selected NBS-encoding R genes from cassava. Black colour represents the gene sequences of the promoter region and downstream regions.

The selected NBS protein sequences were subjected to motif analysis using the MEME analysis tool (http://meme-suite.org/tools/meme). Fifteen conserved motifs were identified and comprised of amino acids residues ranging from 9 to 50 (motifs 15 and 1, respectively). All the 7 cassava NBS-LRR proteins contained motifs 1, 5, 8, 9, 10 and 14 indicating that these motifs are conserved in the NBS-LRRs used in the current study. The conserved motifs were dispersed throughout the protein sequences. Each NBS-encoding protein contained different numbers of conserved motifs ranging from motifs 13 to 14 (Figure 2.4).

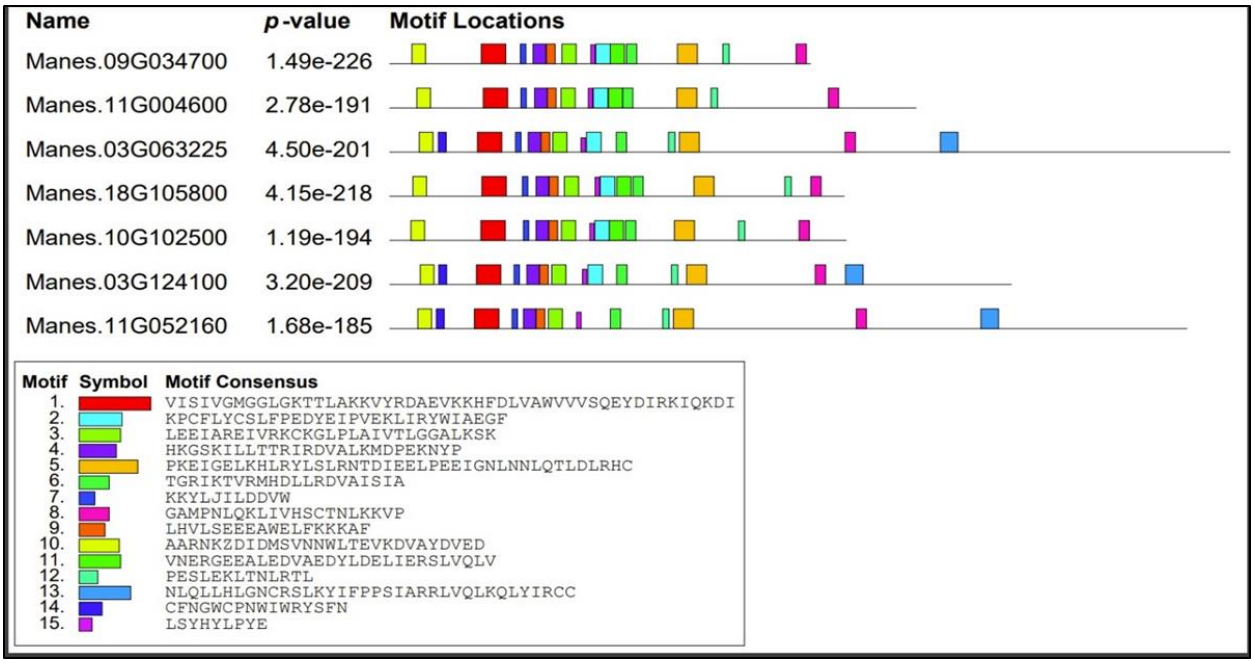


Figure 2.4: Conserved motifs of NBS-encoding R genes in cassava genome.

A total of fifteen motifs were found. Motifs are represented by various colored rectangles. Displayed are logos, motifs, and sequence IDs of preserved motifs. The MEME motif-based sequence analysis tool was used to analyze the conserved motifs. The legend contains motif information.

2.3.3 Homology modeling of selected NBS-LRRs in cassava

Protein predict software was used to predict the secondary structure of the protein, conservation score, disordered and DNA binding regions, and other structural features of a subset of NBS-LRRs found in different cassava varieties. The secondary structure predictions of 7 cassava NBS-LRR proteins showed random coils (43 – 49%) followed by extended strands (29.03 – 36.45%) ad α-helices (12.34 – 15.83%). Notably, no disulfide bond strands and signal peptides were detected in the predicted secondary structure. The protein tertiary structures of the seven cassava NBS-LRRs were constructed (Figure 2.5) based on the predictions from the SWISS-MODEL.

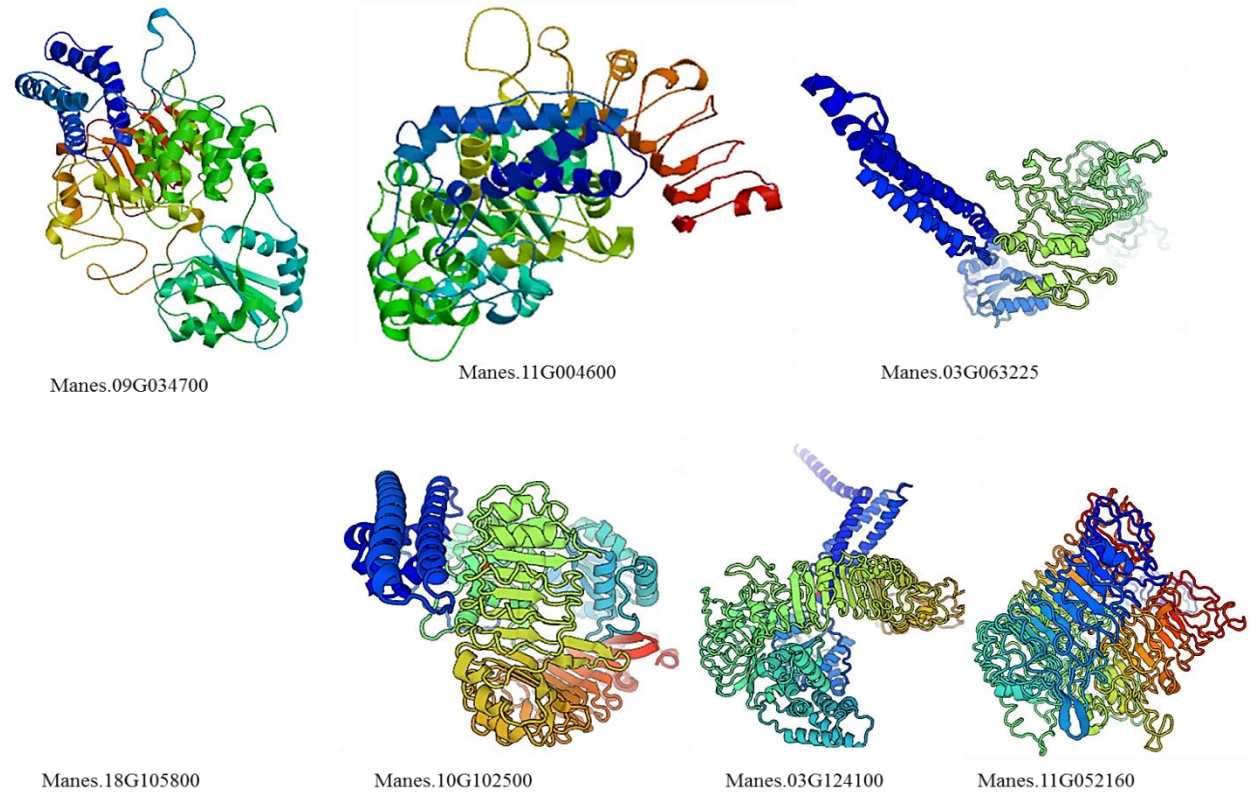


Figure 2.5: Tertiary structure of seven cassava NBS-LRR proteins.

Acid and basic residues are represented by blue and red hues respectively. The protein's predicted binding ligand locations are shown by green colors.

2.3.4 CMD symptoms development and virus detection

Virus indexing by PCR confirmed the absence of the EACMV in the plants of all the 8 varieties before graft-inoculation. Following graft-inoculation of 8 cassava varieties, plants were monitored weekly for CMD symptom development for 10 weeks. The graft success rate was 100% for all the tested cassava varieties. Cassava mosaic disease symptoms were observed on all plants of susceptible cassava varieties from 2 to 10 weeks after grafting (Figure 2.6). The appearance of symptoms varied across cassava varieties. All the mock-inoculated plants did not show CMD symptoms.

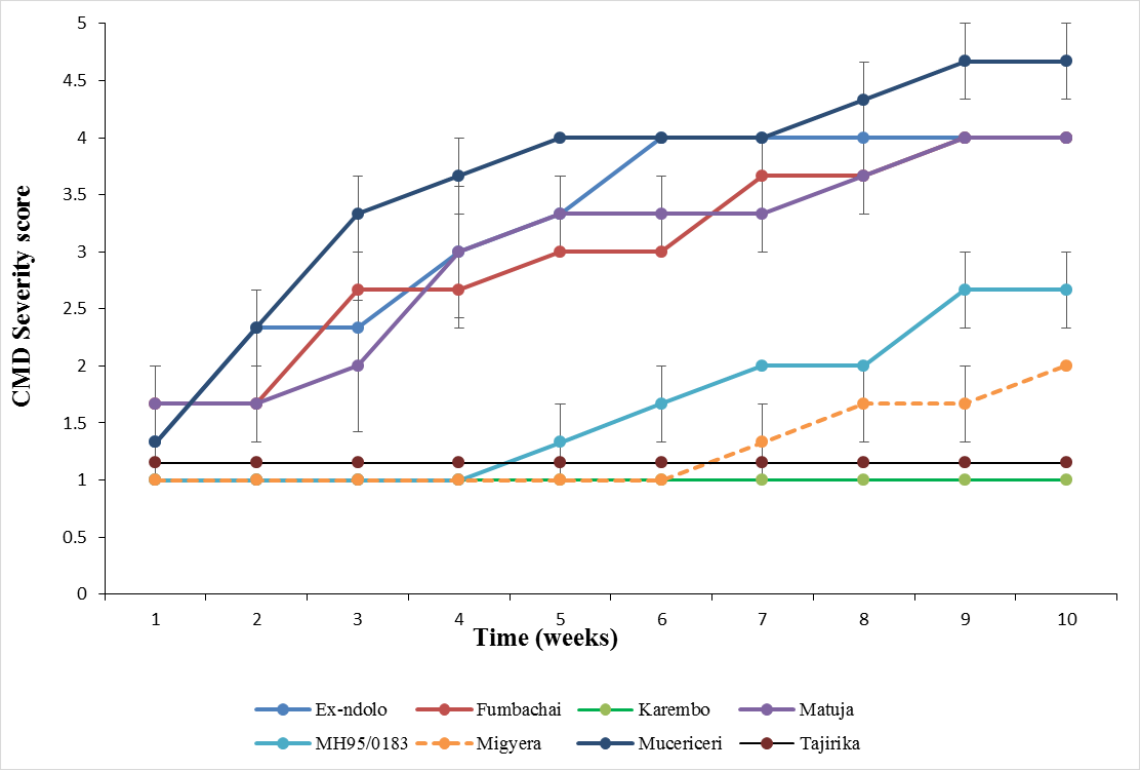


Figure 2.6: CMD symptom severity score of plants of different cassava varieties after graft-inoculation with EACMV.

Error bars represent mean ± standard deviation of five biological replicates and LSD at 5% level.

The symptoms were scored on a disease severity scale of 1 - 5 as described by Houngue *et al*. (2019). Observations in the greenhouse showed that varieties Mucericeri, Fumbachai, Matuja, MH95/0183 and Ex-Ndolo varieties were susceptible and exhibited distinct foliar CMD symptoms that included severe chlorosis, leaf distortion and leaf misshapening (Figure 2.7). A variety of Migyera expressed delayed and mild symptoms which were observed from 7 weeks after grafting. The CMD resistant varieties were Tajirika and Karembo and did not exhibit any CMD symptoms for the 10 weeks of observation in the greenhouse (Figure 2.6). At week 10 after grafting, plants of varieties Mucericeri, Fumbachai, Matuja, MH95/0183 and Ex-Ndolo showed severe leaf symptoms with a mean symptom severity score ranging from 4 to 5 (Figure 2.6). At 10 weeks after infection, mean CMD symptom severity scores were significantly different among the 8 cassava varieties ($p \leq 0.05$). Two varieties had a mean severity score of 1 and six varieties had mean severity scores greater than 1 (Figure 2.6). The varieties Mucericeri, Fumbachai, Matuja and Ex-Ndolo were the most susceptible to EACMV with mean severity scores ranging from 4 to 5 at 10 weeks after grafting.

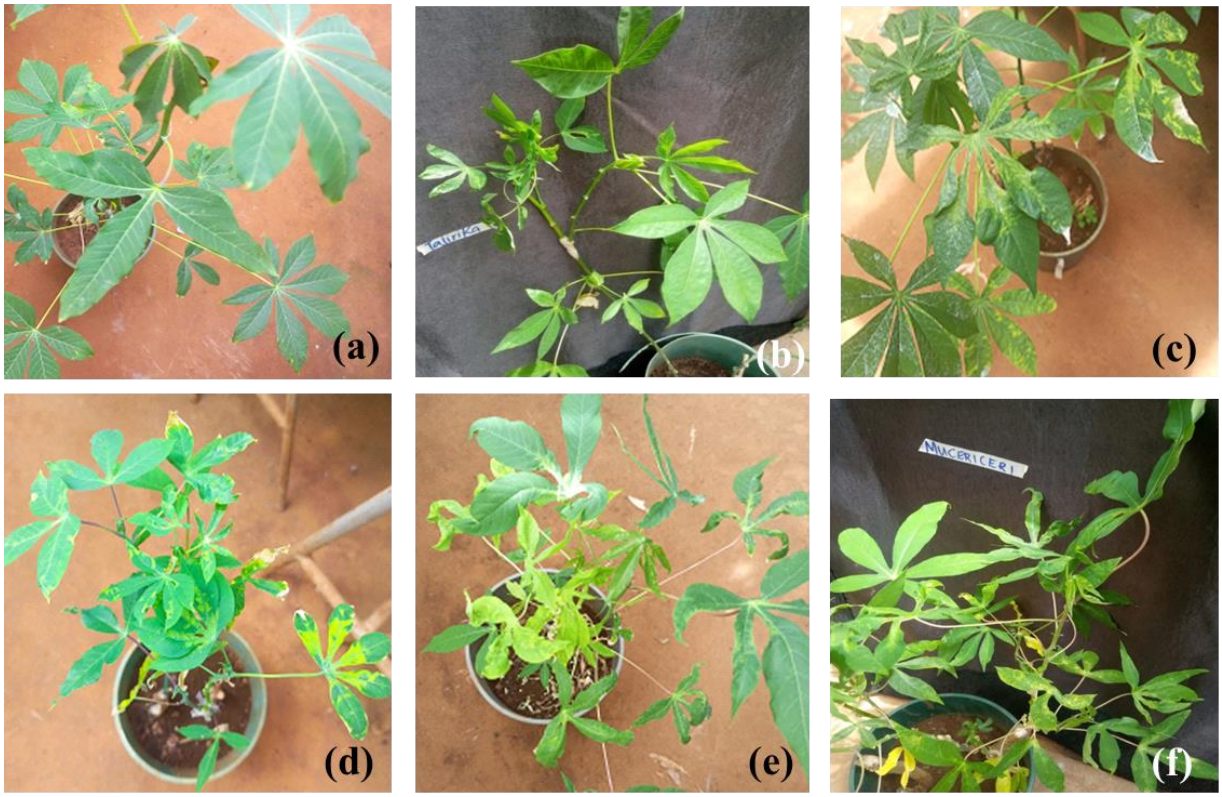


Figure 2.7: CMD symptoms on plants of different cassava varieties after graft-inoculation with EACMV at 70 dpi

(**a**) mock-inoculated Tajirika; (**b**) asymptomatic resistant variety Tajirika; (**c**) symptomatic susceptible variety Matuja showing chlorosis and leaf folding; (**d**) symptomatic susceptible variety Fumbachai showing severe chlorosis and yellowing; and (**e**) and (**f**) symptomatic susceptible variety Mucericeri showing severe chlorosis, yellowing and leaf twisting.

All the grafted plants of the 8 cassava varieties were tested by PCR for the presence of EACMV. PCR analysis revealed amplified products of expected size (bp) for all the plants graft-inoculated for susceptible (Mucericeri and Ex-Ndolo) and tolerant (Migyera) cassava varieties. No amplification products were obtained from plants of resistant cassava varieties (Karembo and Tajirika) graft-inoculated with EACMV-infected plants (Figure 2.8).

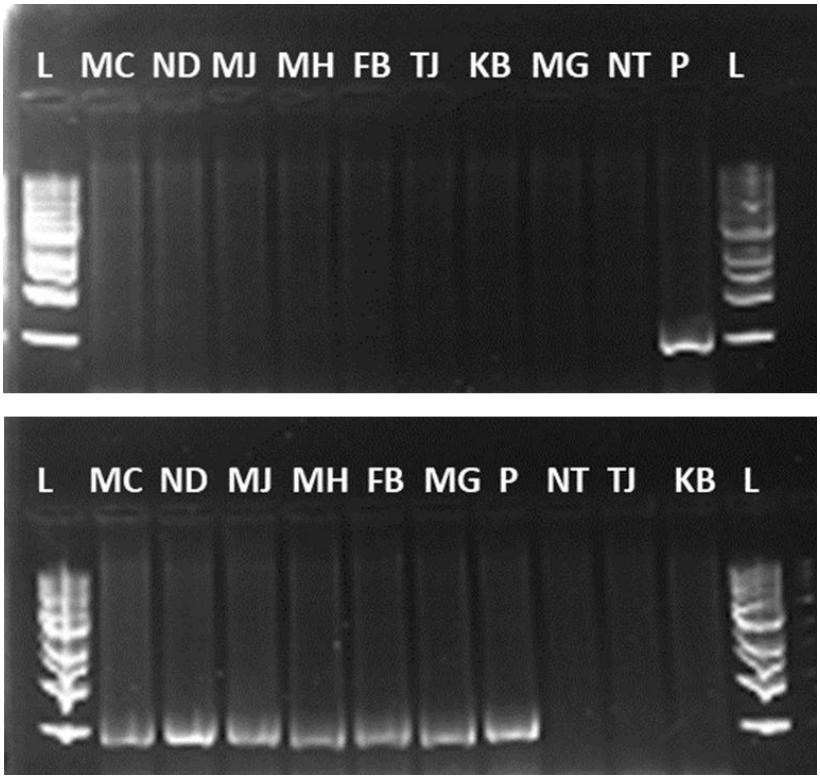


Figure 2.8: Gel image for EAMV detection in EACMV- grafted plants and mock-inoculated cassava plants of eight varieties using PCR.

L represents 1kb hyperladder (Bioline, England), MC, ND, MJ, MH, FB, TJ, KB and MG represent varieties Mucericeri, Ex-Ndolo, Matuja, MH95/0183, Fumbachai, Tajirika, Karembo and Migyera. P represents positive control and NT represents non-template control. The upper lanes represent PCR results for clean plants of cassava varieties before graft-inoculation while the lower lanes represent PCR results for plants of cassava varieties after graft-inoculation.

2.3.5 Expression pattern of NBS-LRR genes in response to EACMV infection

The expression patterns of the selected conserved CC-NBS-LRR genes were analyzed using RT-qPCR in the leaves of two resistant (Karembo and Tajirika), one tolerant (Migyera) and two susceptible (Mucericeri and Ex-Ndolo) cassava varieties after infection with EACMV at 12, 32

and 67 dpi. Differential expression of the seven genes examined in the study was observed at different times between the susceptible and resistant cassava varieties. At 12, 32, and 67 dpi, the expression of all the NBS-LRR genes was generally significantly higher in the resistant cassava varieties compared to the susceptible ones. In susceptible varieties, a general tendency of reduced gene expression was noted. With the exception of Manes.09G034700, all of the genes in the resistant cassava varieties exhibited a general increase in expression within 12 days post-infection from EACMV infection. At 12 dpi, there was little to no change in the gene expression for the susceptible varieties.

The expression of Manes.18G105800 gene examined in this study was significantly higher in the resistant (Karembo and Tajirika) compared to the susceptible (Mucericeri and Ex-Ndolo) cassava varieties at 12-, 32- and 67-dpi (Figure 2.9). After infection with EACMV, the transcript levels of Manes.18g105800 gene in the resistant varieties rapidly increased within 12 dpi and reached a peak at 32 dpi, when the expression was 20.58-fold (for Tajirika) and 13.27-fold (for Karembo) higher that at 0 dpi and then rapidly decreased. In contrast, the expression of Manes.18g105800 gene in the susceptible genotypes did not show any significant increase over time after EACMV infection. Real-time quantitative PCR data revealed that, Manes.11G004600 gene expression was significantly up regulated in resistant varieties (Tajirika and Karembo) in response to EACMV infection after 12 dpi, which continued to increase at 32 and 67 dpi (Figure 2.9). There was no significant change in the expression of Manes.11G004600 gene in the susceptible genotypes over time after infection with EACMV.

The transcript levels of Manes.11G004600 gene in the tolerant variety “Migyera” significantly increased and reached a peak at 12 dpi, when its expression was 3.73-fold higher that at 0 dpi and then there was no significant change over time (12, 32 and 67 dpi) after EACMV infection.

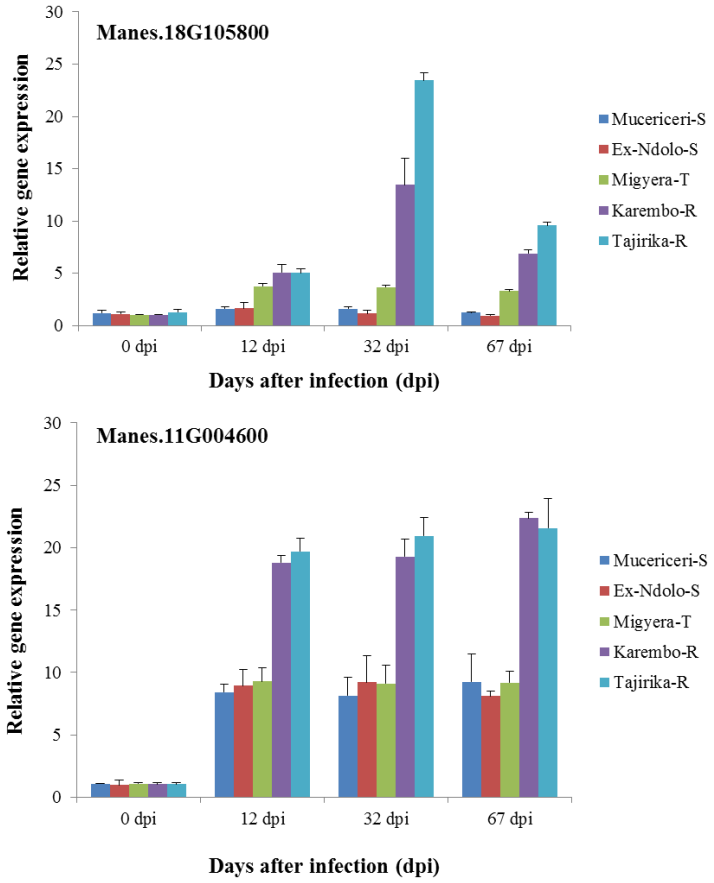


Figure 2.9: Expression profiles of candidate NBS-LRR genes evaluated by real-time PCR in resistant, tolerant and susceptible cassava varieties in response to EACMV infection at 0, 12, 32 and 67 days after infection (dpi).

The 2-ΔΔCT algorithm was utilized to compute the relative gene expression values, and PP2A was utilized as the endogenous reference gene for normalization. The confidence intervals corresponding to three biological replicates, each consisting of three plants, are represented by error bars. According to Tukey's test at a 95% significance level, different letters on the bars indicate significant statistical differences. Following the variety name, the letters R, T, and S stand for resistant, tolerant, and susceptible cassava varieties, respectively.

The expression pattern of Manes.03G063225 gene was consistently high in the resistant varieties at all-time points (12, 32 and 67) after EACMV infection (Figure 2.10). In the tolerant cassava variety "Migyera," the expressions of the gene were low at all-time points (12, 32 and 67) and did not show significant change over time after EACMV infection. Manes.03G063225 gene expression was significantly downregulated in susceptible cassava varieties (Mucericeri and Ex-Ndolo) at 12, 32 and 67 dpi with EACMV as compared to 0 dpi. The expression of Manes.09G034700 gene in the resistant cassava varieties followed an up-down-up trend i.e. the gene expression increased at 12 dpi, decreased at 32 dpi and increased again at 67 dpi after infection with EACMV (Figure 2.10). The highest expression level occurred at 12 dpi, which was 4.55-fold (for resistant variety Tajirika) higher than that at 0 dpi. The expression level rapidly increased within the first 12 days after inoculation, reached its peak at 12 dpi and then rapidly decreased. At 67 dpi, the expression increased further. No significant changes in the Manes.09G034700 transcript levels were observed in susceptible cassava varieties.

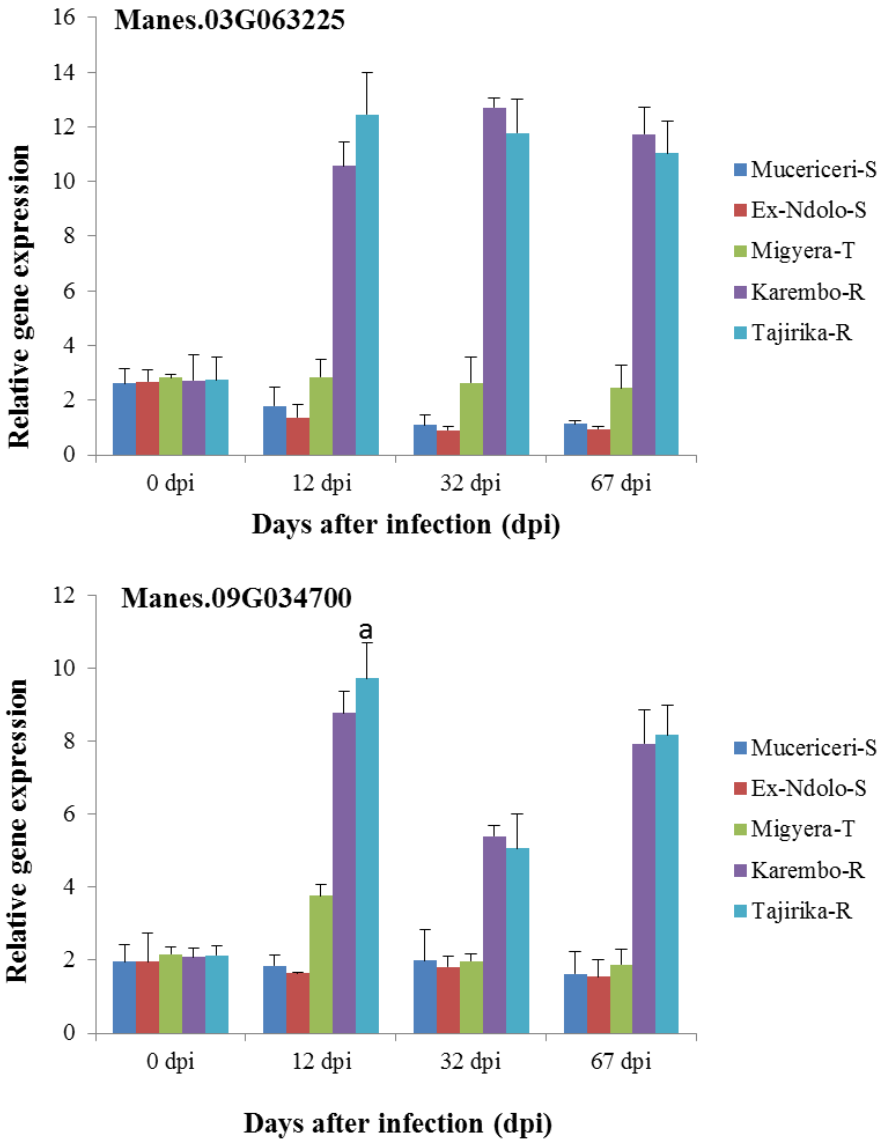


Figure 2.10: Gene expression profiles of candidate NBS-LRR genes evaluated by real-time PCR in resistant, tolerant and susceptible cassava varieties in response to EACMV infection at 0, 12, 32 and 67 days after infection (dpi).

The 2-ΔΔCT algorithm was utilized to compute the relative gene expression values, and PP2A was utilized as the endogenous reference gene for normalization. The confidence intervals corresponding to three biological replicates, each consisting of three plants, are represented by error bars. According to Tukey's test at a 95% significance level, different letters on the bars indicate significant statistical differences. Following the variety name, the letters R, T, and S stand for resistant, tolerant, and susceptible cassava varieties, respectively.

The expression pattern of Manes.010G102500 gene displayed an increased trend in the resistant varieties with increasing time after infection with EACMV (Figure 2.11). The expression in resistant varieties (Tajirika and Karembo) exhibited rapid upregulation from 12 dpi to 67 dpi, while in the tolerant and susceptible varieties there was no significant change. In the susceptible varieties the expressions of the gene was very low in the initial EACMV infection and did not show significant change over time.

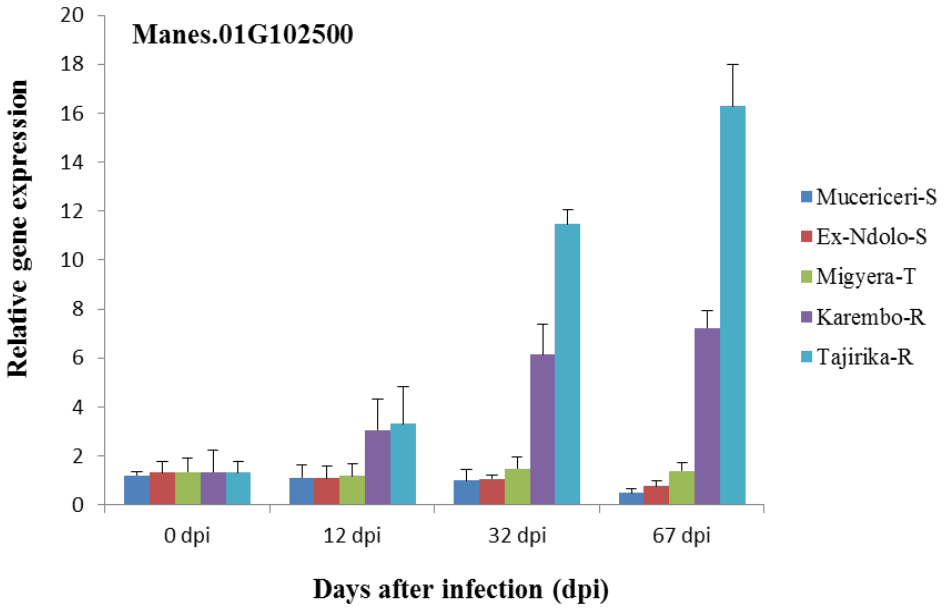


Figure 2.11: Gene expression profiles of candidate NBS-LRR genes evaluated by real-time PCR in resistant, tolerant and susceptible cassava varieties in response to EACMV infection at 0, 12, 32 and 67 days after infection (dpi).

The 2-ΔΔCT algorithm was utilized to compute the relative gene expression values, and PP2A was utilized as the endogenous reference gene for normalization. The confidence intervals corresponding to three biological replicates, each consisting of three plants, are represented by error bars. According to Tukey's test at a 95% significance level, different letters on the bars indicate significant statistical differences. Following the variety name, the letters R, T, and S stand for resistant, tolerant, and susceptible cassava varieties, respectively.

Manes.03G124100 gene expression showed significant differences between resistant and susceptible cassava varieties in response to EACMV infection over time. The expression of Manes.03G124100 gene in the resistant cassava varieties increased until 32 dpi followed by a significant decrease at 67 dpi (Figure 12.12). The maximum (17.16-fold) relative expression was observed in resistant variety Tajirika at 32 dpi with EACMV and susceptible variety Mucericeri showed a minimum (1.07-fold) relative expression at 32 dpi with EACMV. Similarly, the expression of Manes.011G052160 gene in the resistant cassava varieties increased until 32 dpi followed by a significant decrease at 67 dpi (Figure 12.12). Compared with the expression levels at 0 dpi, the expression level of resistant cassava varieties after infection with EACMV was significantly higher than that of the susceptible and tolerant cassava varieties.

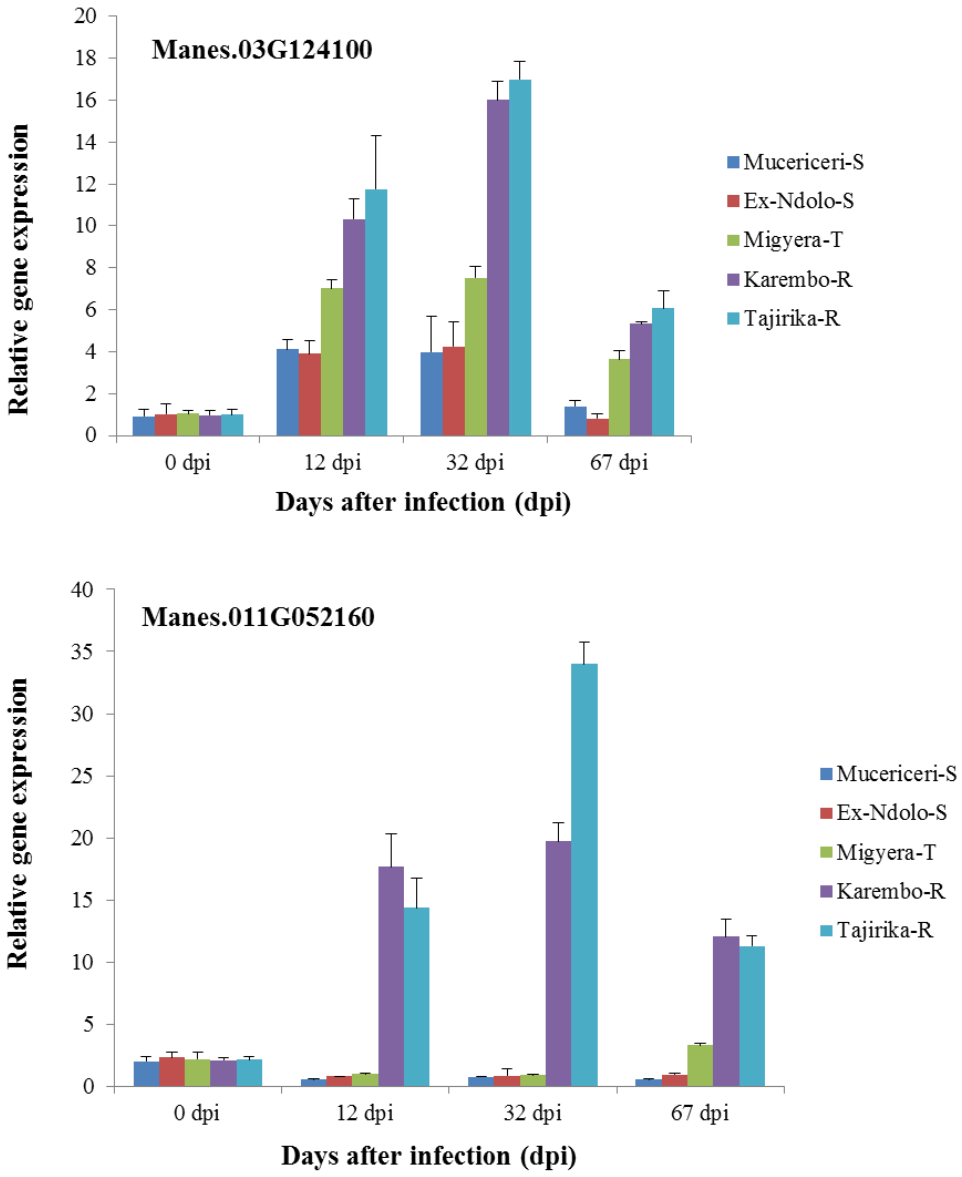


Figure 2.12: Gene expression profiles of candidate NBS-LRR genes evaluated by real-time PCR in resistant, tolerant and susceptible cassava varieties in response to EACMV infection at 0, 12, 32 and 67 days after infection (dpi).

The 2-ΔΔCT algorithm was utilized to compute the relative gene expression values, and PP2A was utilized as the endogenous reference gene for normalization. The confidence intervals corresponding to three biological replicates, each consisting of three plants, are represented by error bars. According to Tukey's test at a 95% significance level, different letters on the bars indicate significant statistical differences. Following the variety name, the letters R, T, and S stand for resistant, tolerant, and susceptible cassava varieties, respectively.

2.4 Discussion

Plant resistance against infections is contingent upon precise identification of pathogen effector proteins generated by plant R genes and their subsequent initiation of a cascade of defensive mechanisms (Fernandez-Gutierrez and Gutierrez-Gonzalez, 2021; Zhang *et al*., 2016). Identified as the largest family of R genes, the NBS-LRR gene family has been extensively studied for its involvement in numerous plant defense mechanisms. The mechanisms by which cassava R genes protect the plant from the EACMV infection is still unknown. The identification, characterization, and functional analysis of the R genes are essential in understanding how cassava develops resistance against EACMV.

The length of the protein sequence, the isoelectric point, and the molecular weights of MeLRRs proteins varied greatly in their physiochemical characteristics. The functional diversification in combating different cassava infections may be linked to this structural variance. The seven MeLRRs genes analyzed in this study are scattered dispersedly throughout the cassava chromosomes, according to the analysis. The number of exons and introns in MeLRRs varies; this structural analysis may be one of the processes driving the evolution of multigene families. NBS-LRR proteins play two crucial roles in defensive response, signal transduction and pathogen detection (Urbach and Ausubel, 2017).

There are an increasing number of cloned NBS-LRR proteins that provide disease resistance from higher plants (Liu *et al*., 2017). TaRCR1 (Zhu *et al*., 2017), ZmNBS25 (Xu *et al*., 2018), GhDSC1 (Li *et al*., 2019), and OsRLR1 (Du *et al*., 2021) are a few of these proteins. According to this research, NBS-LRR expression may be specific to an EACMV infection. Other plant NBS-LRR genes, such as SacMi and ahRRS5, exhibit comparable expression patterns, according to research

by Zhou *et al*. (2018) and Zhang *et al*. (2017). We hypothesized that MeLRR overexpression will help cassava fight EACMV infection, since NBS-LRRs mostly contribute to plant resistance against pathogen infection.

In the current study, the evaluation of cassava varieties for resistance to CMD revealed distinct responses among the tested varieties and significant differences in disease severity scores among varieties. The EACMV infected both susceptible and tolerant cassava varieties as evidenced by the expression of CMD symptoms in plants of these varieties. The tolerant variety Migyera showed delayed symptom development as compared to the susceptible varieties (Mucericeri and Ex-Ndolo). Disease symptoms were not observed in cassava varieties, Tajirika and Karembo demonstrating their resistance to CMD. The findings from this study demonstrate that the resistant cassava varieties (Tajirika and Karembo) to EACMV are promising candidates for future breeding programs. These varieties can also be used as resistance controls for improving susceptible cassava varieties through mutagenesis or genetic modification strategies.

Selected NBS-LRR gene expression patterns after EACMV infection were used to examine the molecular mechanisms behind defense responses in the cassava varieties Tajirika and Karembo. Previous research has shown that geminiviruses upregulate the NBS-LRR gene, and they are known to trigger a particular defense response (Kushwaha *et al*., 2015; Maiti *et al*., 2012). In virus resistance genes in tobacco and potatoes, the function of NBS-LRR in disease resistance against a variety of pathogens has been investigated (Whitham *et al*., 1994; Bendahmane *et al*., 2000). Increased resistance to infections has a favorable correlation with upregulation of NBS-LRR genes (Sett *et al*., 2022). For instance, without impairing plant growth or development, the overexpression of the soybean NBS-LRR gene GmKR3 increased the plant's resistance to many

strains of the soybean mosaic virus (Xun *et al*., 2019). Furthermore, it has been reported that barley and wheat exhibit increased transcript accumulation of NBS-LRR genes in response to barley yellow dwarf viruses (Alquicer *et al*., 2023). The ability of MeNBS-LLR to combat EACMV, however, has not yet been thoroughly investigated or utilized.

To prevent the infection from spreading and multiplying, NBS-LRR-mediated defense systems recognize particular pathogen effectors and trigger downstream defensive signaling pathways. The expression patterns of pathogen-responsive MeNBS-LRR genes in susceptible and resistant cultivars at various stages following EACMV infection were examined in this work. Depending on the variety's resistance and amount of time after virus infection, notable variations in gene expression patterns and symptom severity were observed. The qRT-PCR study revealed that the expression of the seven MeLRRs genes varied across susceptible and resistant cassava cultivars. Higher NBS-LRR gene expression levels were observed in resistant compared to susceptible varieties suggesting that some NBS-LRR genes may be up-regulated and contribute to CMD resistance. The activation of the plant defense system was significantly influenced by the up- and down-regulation of distinct NBS-LRR genes at different times.

The expression levels of the NBS-LRR genes peaked earlier in the resistant varieties, indicating that the aforementioned genes played positive regulatory roles when cassava plants were subjected to EACMV infection. The earlier up-regulation of MeNBS-LRR genes indicated that these genes could likely respond to the disease at the initial stage of infection. The compatible interactions between the susceptible host and the virus leads to the increase in the multiplication of the virus on the host. Incompatible interactions between the resistant varieties and EACMV resulted in disease resistance reaction due to the inhibition of virus multiplication with increased defense responses during the early infection stages. The expression of two genes (Manes.03G063225 and

Manes.11G004600) up regulated constantly from 12 dpi to 67 dpi suggests that they may be involved in longer stages of the disease response. The fact that the selected MeNBS-LRR genes in resistant cassava varieties respond to EACMV infection suggests that their potential overexpression is a strategy to mitigate the negative effects of CMD.

2.5 Conclusion

The study explored various structural characteristics such as conserved motif analysis, exon-intron structure for the selected MeNBS-LRR genes and demonstrated that the MeNBS-LRR genes have a broad range of variations in length, number of exons and introns, molecular weight and isoelectric point. To determine their response against EACMV infection, an experiment was conducted to analyze the transcript accumulation profiles of the seven cassava NBS-LRR genes using specific primers after inoculation with EACMV in five cassava varieties with differential response to CMD. The seven MeNBS-LRR candidate genes are involved in the response of cassava to EACMV infection based on qRT-PCR expression analysis in the resistant and susceptible cassava varieties.

2.6 Recommendations

This study lays the foundation for additional functional research to validate the link between NBS-LRR genes and cassava CMD resistance. In order to create cassava cultivars resistant to CMD, these results should also make marker-assisted breeding easier. Together with their eventual application in the cassava molecular breeding programs, the existing MeNBS-LRRs may also aid in the development and validation of R-gene specific molecular markers. Moreover, investigation on the mode of action of the genes in cassava mosaic disease pathway is also necessary. Additionally, the expression of MeNBS-LRR regulatory components could be thoroughly analyzed and regulated through the use of gene editing tools.

CHAPTER THREE

3.0 ELIMINATION OF CASSAVA MOSAIC GEMINIVIRUSES FROM INFECTED STEM CUTTINGS USING HYDROGEN PEROXIDE, SALICYLIC ACID AND HOT WATER TREATMENT

Abstract

Significant losses in cassava output occur in Africa, South East Asia, and India due to cassava mosaic disease (CMD). The disease is mostly spread by the use of infected plant materials and vector-borne transmission. It is caused by several cassava mosaic geminiviruses (CMGs). Because the disease cannot be managed by common plant protection techniques, infected plants stay infected throughout their vegetative lifecycle. In order to sanitize the planting material contaminated with geminiviruses, it is crucial to create novel and affordable field-based techniques that small-scale farmers may readily implement. In this research, salicylic acid (SA), hydrogen peroxide (H_2O_2), and hot water (HW) treatments were utilized to eliminate geminiviruses (CMGs) from infected cassava stem cuttings. Prior to being introduced to the glasshouse, eight different varieties of CMD-infected stem cuttings underwent pretreatment with varying concentrations of SA (1.25 mM, 2.5 mM, and 5 mM), H_2O_2 (0.5%, 1%, and 1.5%), or HW at temperatures of 50 ºC and 55 ºC at different exposure times. The goal was to evaluate subsequent plant growth and the elimination of geminiviruses. Stem cuttings treated with HW at 50 ºC for 5 minutes and 1% H_2O_2 for 12 hours were found to be the most effective in eliminating CMGs, with respective efficiencies of 81.7% and 77.8%, varying by cassava variety. 65.1% of the cuttings had the viruses eradicated after exposure to 5 mM dose of salicylic acid for 6 hours. In order to address the growing need of small-holder farmers and the cassava sector, the technologies tested herein have the ability to produce virus-free planting materials. It might also help spread virus-free stem cuttings for breeding and conservation initiatives worldwide.

3.1 Introduction

Several biotic factors, such as cassava mosaic disease, have an impact on the productivity and quality of the cassava (*Manihot esculenta*) crop (Mohammed *et al*., 2012; Thresh and Cooter, 2005). Several CMGs (genus Begomovirus, family Geminiviridae) are responsible for the disease. Notable species that wreak havoc on the African continent include the African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV), and South African cassava mosaic virus (SACMV) (Jacobson *et al*., 2018; Zerbini *et al*., 2017). Cassava mosaic disease (CMD) causes green to yellow mosaic foliar symptoms, often with leaf distortion in infected cassava. These morphological alterations in the leaves result in reduced photosynthesis leading to poor development of tuberous roots, which ultimately reduces yields. Storage root yield losses in sub-Saharan Africa have been estimated to be 12 - 23 million tons which is equivalent to a 1.2 to 2.3 billion USD loss annually (FAOSTAT 2020). Estimated yield losses from CMD in susceptible cultivars range from 20 to 95% (Arama *et al*., 2016; Legg *et al*., 2015; Tomlinson *et al*., 2018). Since traditional plant protection methods cannot control the CMD-associated geminiviruses, cultivating healthy vegetable propagation material is essential for sustainable cassava production.

Cassava mosaic geminiviruses are propagated by infected planting material and transmitted/spread by the whitefly vector, *Bemisia tabaci* Gennadius (Macfayden *et al*., 2018, Duraisamy *et al*., 2013; Maruthi *et al*., 2005). The vegetative nature of cassava stem cuttings makes it prone to the dissemination of CMD. Methods to clean cassava mosaic geminivirus infections, such as thermotherapy, and somatic embryogenesis (Mutai *et al.,* 2017; Maruthi *et al*., 2019; Zinga *et al*., 2014; Nakabonge *et al*., 2020) have been developed so far using *in vitro* culture. Unfortunately, these approaches are not cost-effective and therefore often not applicable to small-scale farmers in sub-Saharan Africa (Okori and Nakabonge, 2016). Therefore, field-based methods to sanitize

geminiviruses-infected stem cuttings used as planting material need to be developed to meet the increasing demand for disease-free seeds.

There are various plant viruses whose replication is known to be inhibited by high temperatures. According to studies conducted by Oltrepalska-Steplowska *et al*. (2015) and Chellappan *et al*. (2005), the application of high-temperature treatments is successful in getting rid of viruses in plants that are vegetatively propagated using *in vitro* culture. According to Nangonzi *et al*. (2016), the high temperatures used in heat therapy prevent viral replication, thus preventing their survival. Hot water is a less complex technique that involves less expensive laboratory equipment but is effective in removing viruses from plants. Hot water treatment has been reported to be effective in eliminating viruses from plants (Abbas *et al*., 2016; Nangonzi *et al*., 2016; Ling, 2010).

Phytohormones are induced in plants upon pathogen attack to enhance resistance (Collum and Culver, 2016; Ghosh and Chakraborty, 2021; Gupta *et al*., 2022; Zhao and Li, 2021; Zhong *et al*., 2021. Using this knowledge, scientists have enhanced the resistance of plants to infections by exogenously administering phytohormones. One such phytohormone that causes systemic and localized hypersensitivity response (HR) is salicylic acid, whose accumulation is boosted by a number of incompatible host interactions (Jovel *et al*., 2011; Baebler *et al*., 2014). When a pathogen attacks a plant, the SA pathway is activated. This starts the HR, prevents viral movement and replication, and reduces the build-up of coat proteins (Murphy *et al*., 2020). Reactive oxygen species (ROS) buildup and the synthesis of antimicrobial chemicals are some other aspects of defensive reactions. Hydrogen peroxide as an ROS has two major functions. First, it inhibits the pathogen by inducing localized cell death. Secondly, it produces signals that activate genes associated with pathogenesis and antioxidants in nearby plant tissues. As ROS generated when

pathogens infect plants, H_2O_2 promotes gene expression and produces enzymes required for plant defense against viruses (Mejía-Teniente *et al*., 2019; Radwan and Ismail, 2019).

The aim of the present study was to evaluate the protective effects of cassava plants against cassava mosaic geminiviruses infection by exogenous treatment of stem cuttings used as planting material with SA, H_2O_2, and HW.

3.2 Materials and Methods

3.2.1. Plant material

Stem cuttings of 9-month-old plants of eight cassava varieties exhibiting CMD symptoms were collected from the fields at Kenya Agricultural and Livestock Research Organization (KALRO), Kakamega station in western Kenya in November 2022. The cuttings were 15 cm long and were cut from the middle-top part of the plants. The collected varieties were MM96/5280, Fumbachai, Nanzala, Matuja, Nyadai, Serere, Magana and Abarobaka. Before collection of the stem cuttings for virus elimination treatments, leaves from each variety were sampled and used for virus indexing to confirm the CMD status of the plants. The cassava plants collected as a source of stem cuttings for the experiment had a symptom severity score of 4 based on Houngue *et al*. (2019) severity score scale.

3.2.2 Confirmation of cassava mosaic geminiviruses in plants of the selected cassava varieties.

By utilizing virus-specific primers for the detection of ACMV and EACMV, PCR was used to confirm the presence of the CMGs in the selected cassava varieties (Table 1) (Alabi *et al*., 2008). The CTAB method was used to extract whole genomic DNA (Osena *et al*. (2017). After resuspension of the DNA pellet in nuclease-free molecular grade water, the DNA concentration

was determined using a Fisher Thermo Scientific NanoDrop 2000 spectrophotometer and diluted to 100 ng/µl.

After the DNA extraction, PCR was conducted on the samples using specific primers to detect ACMV and EACMV (refer to Table 1). For the PCR reaction, 12.5 µl of Quick-Load Taq 2× master mix (New Englands Biolabs), 0.5 µl of 10 µM each of forward and reverse primers, 2 µl of DNA template, and 9.5 µl of nuclease-free molecular-grade water were used, resulting in a final reaction volume of 25 µl. The PCR was carried out using a conventional thermocycler from Eppendorf AG 22331, Hamburg, Germany. The temperature profile for the PCR process was as follows: Initial denaturation at 95°C for 3 mins, 30 cycles of amplification at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final elongation step at 72 °C for 10 min. The amplicons obtained from the PCR were separated through 1% agarose gel electrophoresis, stained with 0.2× GelRed nucleic acid dye (Biotium, Fremont, CA, USA), and observed under UV light. The gel documentation system (Azure C200, Biosystems, USA) was used to take the gel images. The stem cuttings of ACMV- and EACMV-positive plants were used for SA, H_2O_2, and HW treatment experiments.

Table 3.1: Primer pairs used for the amplification of ACMV and EACMV

Primer	Sequence (5′ – 3′)	Target	Expected size (bp)	Virus species	Reference
EACMV A2469s EACMV A391c	TGGAGATGAGGCACCCCATC TCCTCCGCACCTTGGATACG	DNA-A	722 bp	EACMV	Ferguson *et al.*,2020
ACMV-AL1/F ACMV-ARO/R	GCGGAATCCCTAACATTATC GCTCGTATGTATCCTCTAAGGCCTG	DNA -A	1030 bp	ACMV	Alabi *et al*.,2008
CMBRep F ACMVRep R	CRTCAATGACGTTGTACCA CAGCGGMAGTAAGTCMGA	REP gene	368 bp	ACMV	Alabi *et al*., 2008
CMBRep F EACMVRep R	CRTCAATGACGTTGTACCA GGTTTGCAGAGAACTACATC	REP gene	650 bp	EACMV	Alabi *et al*., 2008

3.2.3 Salicylic acid, hydrogen peroxide and hot water treatments of CMD-infected stem cuttings

Stem cuttings of 9-month-old plants of varieties MM96/5280, Fumbachai and Nanzala were used for SA treatments. Varieties Matuja, Nyadai and Serere were used for H_2O_2 treatment whereas varieties Magana, Abarobaka and Nyadai were used for HW treatment. Cassava varieties were immersed in different concentrations of SA (1.25 mM, 2.5 mM and 5 mM) for 6, 12 and 24 h; different concentrations of H_2O_2 (0.5%, 1% and 1.5%) for 6, 12 and 24 h and different HW temperatures (50 ºC and 55 ºC) for 5- and 10-min. The SA and H_2O_2 treatments were carried out at room temperature. Stem cuttings (approximately 4 – 5 nodes and 9 biological replicates) were subjected to each treatment combination (concentration/temperature × exposure time) and each treatment combination was replicated three times. Sterile distilled water was used as a control. All

treatment combinations and control stem cuttings were planted in plastic pots in a completely randomized design with each treatment combination having a total of 9 stem cuttings and the experiments were replicated three times. Thus, a total of 27 stem cuttings were used for each treatment combination (concentration/temperature × exposure time).

3.2.4 Establishment of SA, H_2O_2 and HW treated stem cuttings and phenotypic analysis of established plants in the glasshouse

The stem cuttings treated with SA, H_2O_2 and HW were planted in plastic pots (Kenpoly, Kenya) containing sterilized soil mixed with sterilized farmyard manure in the ratio of 1:3 (v/v) and placed under controlled conditions (28 ºC and 70% relative humidity). Survival was evaluated 2 weeks after treatments by counting the number of stem cuttings that sprouted. Data regarding the number and height of sprouted shoots were recorded at 2, 4, 6 and 8 weeks after sprouting of SA, H_2O_2 and HW treated stem cuttings. Virus severity scores of sprouted leaves were performed using a severity scale of 1 - 5 according to Houngue *et al*. (2019)

3.2.5 Detection of cassava mosaic geminiviruses (ACMV and EACMV) in sprouted plants from SA, H_2O_2 and HW treatments

Virus elimination in 8-week-old cassava plants in the glasshouse was evaluated by PCR analysis using virus-specific primers (Alabi *et al*., 2008). The three top most leaves that exhibited CMD symptoms were sampled for genomic DNA extraction and subsequent PCR analysis as described earlier. Results were analyzed by agarose gel electrophoresis on a 1% agarose gel in a 1× TAE buffer and visualized with UV transilluminator after staining with 0.2× GelRed (Biotium, Fremont, CA, United States).

3.2.6 Analysis of hydrogen peroxide and lipid peroxidation

Newly emerged leaves of plants without CMD infection after treatment of stem cuttings with SA, H_2O_2 and HW and non-treated control plants with CMD infection were analyzed for the levels of H_2O_2 and malondialdehyde (MDA) after 8 weeks in the glasshouse. As described by Velikova *et al*. (2000), the optical density was evaluated using potassium iodide to determine the amount of H_2O_2. Using a mortar and pestle, 0.5 g of leaves were homogenized in 2 ml of ice-cold 0.1% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged at 10,000 rpm for 30 min at 4°C. 1 ml of 1 M potassium iodide and 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) was added to 0.5 ml of the supernatant. The absorbance was measured at 390 nm, and the amount of H_2O_2 was estimated using the extinction coefficient of 0.28/mM/cm and expressed as micromoles per gram fresh weight (μmol/g FW). The accumulation of H_2O_2 was carried out in three randomly chosen biological replicates for each treatment combination (concentration × exposure time), and the experiment was repeated three times. Three technical replicates (using three top most leaves) were used for each biological replicate for each treatment combination.

The level of MDA produced using the TBA-based method as documented by Hodges *et al*. (1999) and Chen and Gallie (2006) was employed to assess lipid peroxidation. 0.1 g leaf sample was homogenized in 2 ml of a 0.1% (w/v) trichloroacetic acid (TCA) solution on ice. Following a 5-minute centrifugation of the suspension at 10,000 rpm, 0.5 ml of the supernatant was collected. Subsequently, 1 ml of 20% (w/v) TCA with 0.5% (w/v) TBA was added to the supernatant, incubated at 95 °C in a water bath for 30 min, and promptly cooled on ice. The solution was centrifuged for 10 min at 10,000 rpm, and the absorbance readings at 532 nm and 600 nm were taken. The absorbance at 600 nm was subtracted from the absorbance at 532 nm to compensate for non-specific turbidity. The MDA concentration was then determined using its extinction

coefficient of 155 mM-1 cm-1. For every treatment combination (exposure time × concentration), three biological replicates were randomly chosen, and the experiment was performed three times to quantify MDA. For every biological replicate for every treatment combination, three technical replicates (using the top three leaves) were used.

3.2.7 Extraction and determination of antioxidant enzyme activities

The antioxidant enzyme activities were determined in fresh leaves of plants without CMD infection after treatment of stem cuttings with SA, H_2O_2 and HW and non-treated control plants with CMD infection. The extraction procedures of plants with all treatment combinations and non-treated control were performed in three randomly selected biological replicates and the experiments were repeated three times. Three technical replicates (using three top most leaves) were used for each biological replicate for each treatment combination. Using a pestle and mortar, 0.5 g of leaf samples (three technical replicates) were homogenized in 2 ml of buffer containing 1% w/v polyvinylpyrrolidone (PVP), 0.2 mM EDTA, and 100 mM potassium phosphate buffer at pH 6.8 on ice. The mixture was then centrifuged for 20 min at 4 °C at 10,000 rpm. Following centrifugation, the enzyme activity of ascorbate peroxidase, peroxidase, and catalase in the supernatants was measured.

Ascorbic acid oxidation was measured and the change in absorbance at 290 nm was recorded to determine the activity of ascorbate peroxidase (APX). A reaction buffer containing 0.5 mM H_2O_2, 0.2 mM Tris/HCl buffer (pH 7.8), and 0.25 mM ascorbic acid was combined with 10 µl of leaf extract. According to Nakano and Asada (1981), the extinction coefficient (2.8 mM^{-1} cm^{-1}) of ascorbate was used to compute the APX activity.

The catalase (CAT) activity was measured using the method outlined by Cakmak *et al*. (1993). 50 µl of the enzyme extract was mixed with 3 ml of the reaction buffer, consisting of 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2. The reduction in absorbance at 240 nm over 1 min due to H_2O_2 breakdown was utilized to determine the CAT activity. The activity was calculated based on the H_2O_2 extinction coefficient (40 mM^{-1} cm^{-1}).

To measure peroxidase (POD) activity, 50 µl of leaf homogenate was combined with 2 ml of the reaction mixture, comprising 25 mM guaiacol, 25 mM H_2O_2, and 50 mM sodium acetate buffer (pH 7). The absorbance at 470 nm, which increases due to the formation of tetra guaiacol (with an extinction coefficient of 26.6 mM^{-1} cm^{-1}), was measured to determine the POD activity. The peroxidase activity was expressed in units g^{-1}FW.

3.2.8 Data analysis

The data were recorded and entered in Excel spreadsheet for statistical analysis. Data on severity score and plant growth were subjected to three-way ANOVA whereas data on oxidative stress markers, antioxidant enzyme activities, survival percentage, disease incidence, virus elimination efficiency were subjected to two-way ANOVA using GenStat software version 15. Comparisons of the means were done using Tukey HSD test at 5% probability level. Graphs and tables with the means were generated using the Genstat software.

3.3 Results

3.3.1 Detection of cassava geminiviruses in plants of selected cassava varieties for sanitation experiments

The virus status of the eight cassava varieties was screened using PCR and the PCR products visualized by agarose gel electrophoresis (Fig. 3.1a). The expected amplicon size was 368 bp for

ACMV and 650 bp for EACMV. Of the leaf samples tested, varieties Matuja, Magana, MM96/5280, Nanzala and Abarobaka were co-infected with ACMV and EACMV, variety Serere was singly infected with EACMV, whereas varieties Nyadai and Fumbachai were singly infected with ACMV (Fig. 3.1a). The stem cuttings of ACMV and EACMV positive plants were used for SA, H_2O_2 and HW treatments for elimination of the CMGs.

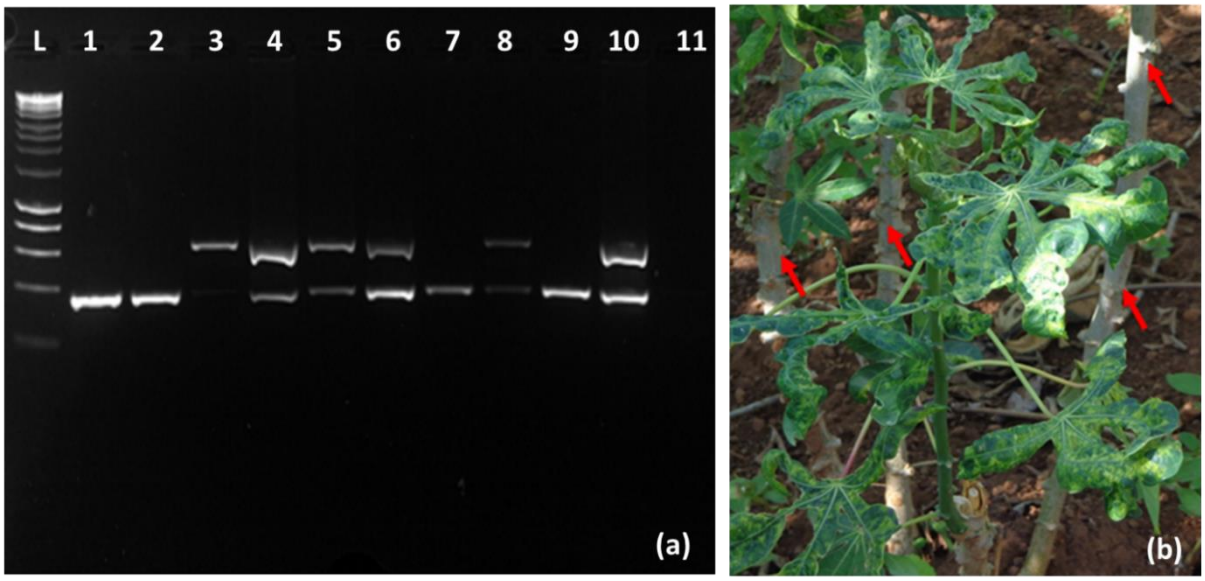


Figure 3.2 Agarose gel electrophoresis showing PCR amplification of DNA from leaves of cassava mosaic virus disease (CMD) infected plants of 8 cassava varieties used for phytosanitation and representative CMD-infected cassava plant showing stem cuttings used for sanitation experiment.

(**a**), Detection of ACMV and EACMV in infected cassava plants. L represents 1 kb DNA Hyperladder (Bioline, London, England); 1 to 6 represents samples from plants of varieties Matuja, Nyadai, Serere, Magana, Abarobaka and MM96/5280, respectively; 7 and 9 represent Fumbachai and 8 represents Nanzala. Lane 10 represents a positive control for EACMV and ACMV while lane 11 represents a non-template control. (**b**), Cassava plants in the field showing source plants of stems (red arrows) used for sanitation.

3.3.2 Effect of H_2O_2, SA and HW treatments on survival rates and plant growth performance of established plants

The survival rate in plants from stem cuttings treated with SA at 1.25 mM for 6 h was the highest ranging from 66.7% to 100%, while from those treated with SA at 5 mM for 24 h had the least survival rate ranging from 22% to 44.4% (Table 3.2) As the concentration and exposure time to SA increased, the survival percentage significantly decreased for all the tested cassava varieties (Table 3.2). The length of the stems of the sprouted plants for all cassava varieties was reduced following SA treatment at all concentration and exposure times. The number of shoots significantly decreased in plants obtained from stem cuttings treated with SA compared to the non-treated controls ($P \leq 0.05$) (Table 3.3).

The survival percentage of plants from stem cuttings treated with 0.5% H_2O_2 for 6 and 12 h was the highest survival, ranging from 87.7% to 100% (Table 3.2). Plants from stem cuttings treated with H_2O_2 for 24 h had the lowest survival percentage ranging from 33.3% to 55.6% for all the three cassava varieties. As the concentration and exposure period of H_2O_2 increased, the survival percentage significantly decreased (Table 3.2).

The length of the stem was reduced in plants from stems treated with H_2O_2 compared to their controls across all the varieties. The number of shoots was significantly reduced ($P \leq 0.05$) in plants sprouted from stem cuttings treated with H_2O_2 compared to the non-treated controls (Table 3.3).

The highest survival percentage of between 77.8% and 100% was recorded in sprouted plants from stem cuttings of all the three cassava varieties treated with HW at 50 ºC for 5 min. The lowest survival percentage of between 44.4% and 55.6% was recorded in sprouted plants from stems

treated with HW at 55 ºC for 10 min for all the three varieties (Table 3.2). As the water temperature increased, the survival rate of sprouted plants was significantly compromised ($P \leq 0.05$). Overall, sprouted plants from stem cuttings treated with HW at 55 ºC showed a highly significant reduction in the length of the stems compared to those treated with HW at 50 ºC (Table 3.3). Plants from stem cuttings treated with HW at 55 ºC showed the highest significant decrease in the number of shoots compared to those treated with HW at 50 ºC (Table 3.3).

Table 3.2 The survival percentages of sprouted plants from stem cuttings treated with H_2O_2, SA and HW at different concentrations/temperature and exposure times.

Variety	Time (h)	H_2O_2 conc. (%)	0	0.5	1	1.5
Matuja	6			88.9±11.1	88.9±0	77.8±0
	12			88.9±6.4	81.5±9.8	66.7±6.4
	24			44.4±6.4	55.6±6.4	44.4±6.4
Control						
Nyadai	6			88.9±6.4	66.7±6.4	55.6±11.1
	12			87.7±0	66.7±11.1	66.7±5.8
	24			44.4±6.4	55.6±5.6	44.4±0
Serere	6			100±0	55.6±6.4	88.9±6.8
	12			100±0	55.6±5.8	66.7±12.8
	24			33.3±6.4	55.6±6.4	33.3±6.4
Control	0		100			
Variety	Time (h)	SA conc. (mM)	0	1.25	2.5	5
Fumbachai	6			66.7±6.4	66.7±0	66.7±6.4
	12			55.6±6.4	77.8±6.4	55.6±6.4
	24			55.6±0	44.4±5.3	44.4±6.4
MM96/5280	6			77.8±11.1	77.8±11.1	77.8±6.4
	12			66.7±6.4	66.7±12.8	66.7±11.1
	24			55.6±12.8	55.6±11.1	33.3±6.4

Nanzala	6			100±0	66.7±0	77.8±11.1
	12			77.8±11.1	44.4±6.4	55.6±6.4
	24			44.4±0	33.3±6.4	22±0
Control	0		100±0			
Variety	Time (min)	HW Temp. (°C)	0	50	55	
Aborabaka	5			100±0	77.8±11.1	
	10			88.9±6.42	44.4±6.42	
Magana	5			81.5±9.8	55.6±12.8	
	10			88.9±11.1	55.6±11.1	
Nyadai	5			77.8±0	55.6±6.42	
	10			66.7±6.42	55.6±11.1	
Control	0		100±0			

The experiments were initiated using 9 stem cuttings for each treatment combination (concentration/temperature × exposure time) and the experiments were replicated three times.

Table 3.3 The growth parameters (stem length and number of shoots) of sprouted plants from stem cuttings treated with H_2O_2, SA and HW at different concentrations and exposure times

			Stem length (cm)				No. of shoots			
Variety	Time (h)	H_2O_2 conc. (%)	0	0.5	1	1.5	0	0.5	1	1.5
Matuja	6			2.23±0.48	2.19±0.85	1.98±0.87		2.94±0.39	1.97±0.47	1.97±0.4
	12			3.96±0.38	8.63±0.58	7.15±0.61		4.36±0.21	6.11±0.24	4.72±0.55
	24			1.46±0.36	6.23±0.71	5.37±1.03		1.22±0.27	4.42±0.42	3.5±0.56
Control			14.26±1.1				6.86±0.29			
Nyadai	6			3.1±0.27	2.75±0.92	3.53±0.35		2.86±0.36	2.61±0.4	3.36±0.37
	12			3.02±0.62	5.3±0.54	4.33±0.46		3.47±0.39	4.31±0.3	3.31±0.37
	24			0.49±0.46	5.67±0.58	1.48±0.59		0.5±0.2	3.08±0.43	1.89±0.38
Control			8.46±0.67				4.97±0.3			
Serere	6			1.96±0.13	3.29±0.4	3.89±0.2		2.64±0.38	2.69±0.48	3.47±0.44
	12			2.23±0.32	4.41±0.68	5.63±0.62		2.63±0.38	3.94±0.41	4.17±0.5
	24			0.38±0.36	2.23±0.64	0.4±0.88		0.53±0.16	2.83±0.39	0.39±0.22
Control	0		8.2±0.96				5.42±0.29			
Variety	Time (h)	SA conc. (mM)	0	1.25	2.5	5	0	1.25	2.5	5
Fumbachai	6			3.66±0.55	3.48±0.56	4.11±0.78		4.42±0.40	3.33±0.47	3.86±0.43
	12			2.72±0.68	1.77±0.43	1.7±0.45		2.81±0.54	2.14±0.41	2.42±0.47
	24			0.29±0.1	0.49±0.2	0		0.81±0.29	0.72±0.31	0

Control				17.68±1.9			5.69±0.37			
MM96/5280	6			1.19±0.22	0.69±0.21	3.42±0.74		2.06±0.35	1.25±0.32	2.94±0.40
	12			1.04±0.24	1.01±0.27	3.6±0.85		1.58±0.36	1.78±0.45	2.89±0.44
	24			0.23±0.1	0.41±0.26	2.37±0.8		0.61±0.23	0.56±0.25	1.33±0.38
Control			4.16±0.79				3.08±0.34			
Nanzala	6			3.51±0.57	7.09±1.26	7.18±1.09		3.92±0.34	4.42±0.48	5.31±0.33
	12			5.19±0.63	5.14±0.76	3.02±0.67		4.19±0.39	4.72±0.46	2.78±0.48
	24			4.56±0.76	4.61±0.84	2.94±0.54		3.50±0.40	3.53±0.47	3.25±0.47
Control	0		10.59±0.82				5.81±0.26			
Variety	Time (min)	HW Temp. (°C)	0	50	55		0	50	55	
Aborabaka	5			1.56±0.24	0.91±0.21			2.44±0.34	1.83±0.4	
	10			0.27±0.1	0.24±0.11			0.86±0.28	0.53±0.21	
Control			4.46±0.72				3.58±0.39			
Magana	5			4.83±0.5	1.24±0.34			4.75±0.17	1.44±0.38	
	10			2.72±0.54	1.7±0.44			2.53±0.38	1.47±0.36	
Control			5.47±0.58				4.67±0.30			
Nyadai	5			4.31±0.72	4.13±0.8			2.75±0.38	2.86±0.46	
	10			5.3±0.74	1.89±0.5			3.61±0.35	1.58±0.34	
Control	0		8.46±0.67				4.97±0.30			

A total of 9 stem cuttings were used for each treatment combination and the experiments were replicated three times. Thus, a total of 27 stem cuttings were used for each treatment combination (concentration/temperature × exposure time).

3.3.3 Symptomatology of sprouted plants from H_2O_2, SA and HW treated stem cuttings.

Characteristic CMD symptoms including chlorosis, leaf distortion, leaf curling/misshapen leaf blades were observed in all the non-treated control plants of all the cassava varieties (Fig. 3.2, 3.3 and 3.4). The onset of minor CMD symptoms in sprouted plants from stem cuttings treated with H_2O_2, SA and HW were delayed compared to the non-treated controls.

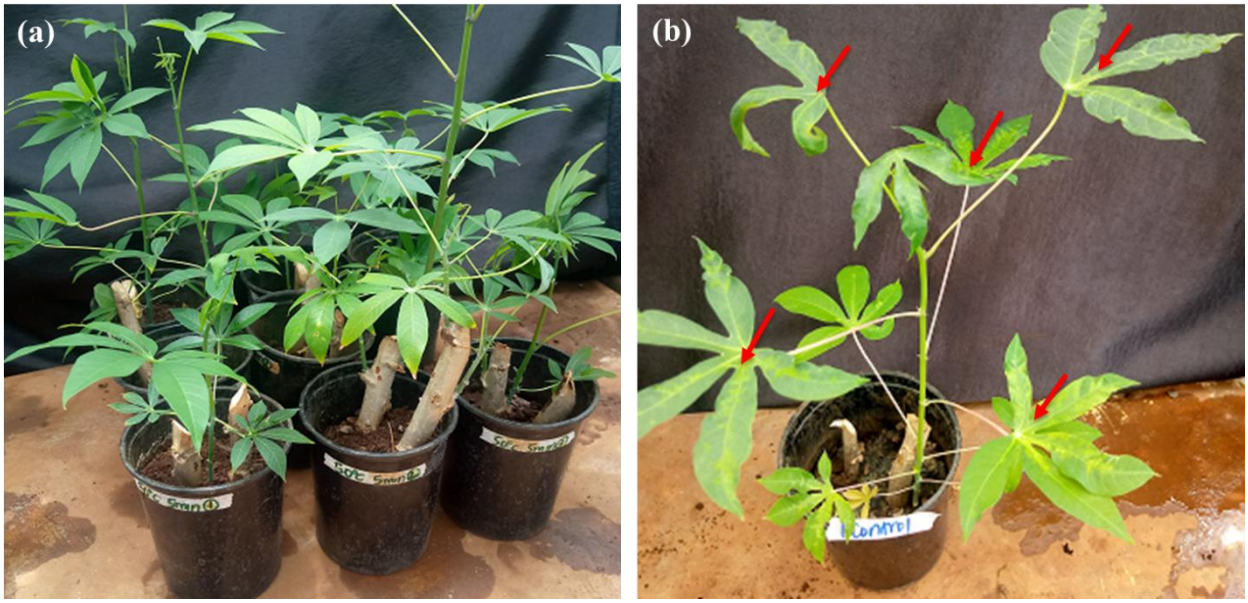


Figure 3.2 Cassava plants established in plastic pots after SA treatments and symptomatic CMD-infected non-treated control plants.

(**a**) Asymptomatic cassava plants after treatment with 5 mM SA for 6 h; (**b**) characteristic CMD symptoms (severe/mild leaf chlorosis, leaf distortion and twisting) on non-treated control plants (plants treated with distilled water).

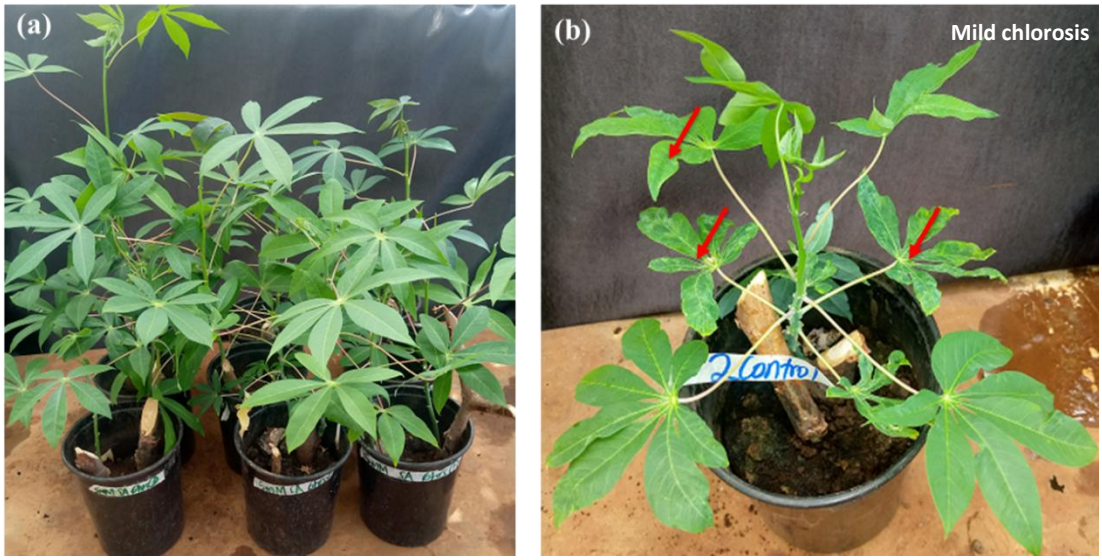


Figure 3.3 Cassava plants established in plastic pots after treatment with H_2O_2 treatments and symptomatic CMD-infected non-treated control plants.

(**a**) Asymptomatic cassava plants after treatment with 1% H_2O_2 for 12 h; (**b**) characteristic CMD symptoms (severe/mild leaf chlorosis, leaf distortion and twisting), on non-treated control plants (plants treated with distilled water).

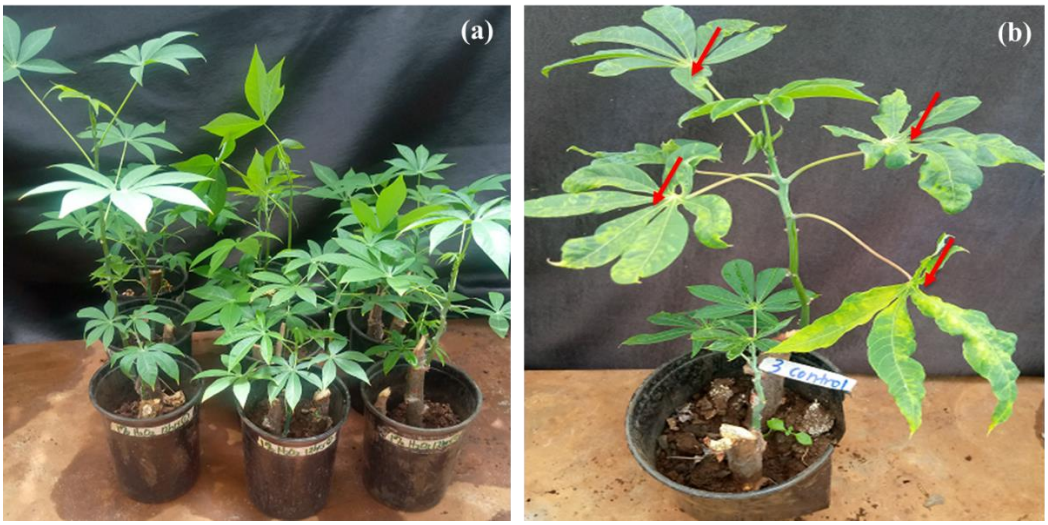


Figure 3.4 Cassava plants established in plastic pots after treatment with HW and symptomatic CMD-infected non-treated control plants.

(**a**) Asymptomatic cassava plants after treatment with HW at 50 ºC for 5 min (**b**) characteristic CMD symptoms (severe/mild leaf chlorosis, leaf distortion and twisting) on non-treated control plants (plants treated with distilled water at room temperature).

3.3.4. Cassava mosaic disease severity in sprouted plants from SA, H_2O_2 and HW treated stem cuttings

The disease symptoms severity score was lowest in sprouted plants from stem cuttings treated with 1% H_2O_2 for 12 h (Fig. 3.5b). Sprouted plants from 5 mM SA-treated stem cuttings for 6 h showed significant reduction in symptom severity score compared to the non-treated controls for all the varieties (Fig. 3.5a). The CMD symptom severity score was significantly reduced in all plants of stem cuttings treated with hot water compared to the non-treated controls (Fig. 3.5c).

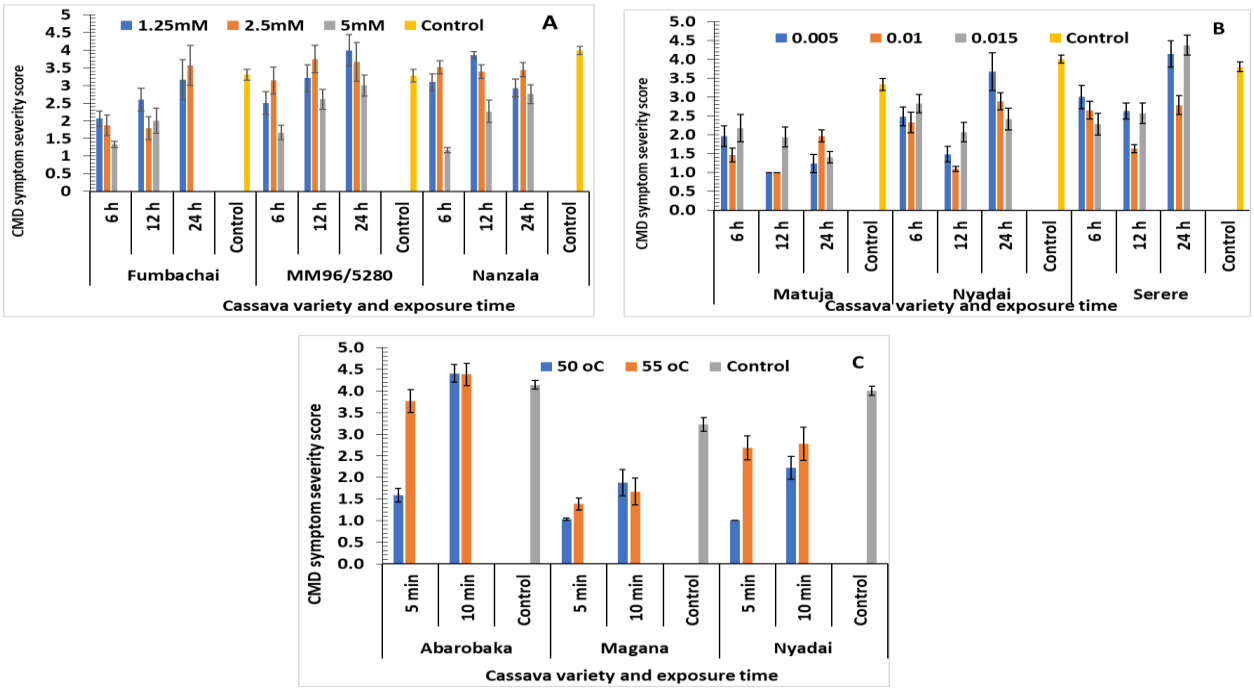


Figure 3.5 CMD severity scores of sprouted plants of different cassava varieties obtained from stem cuttings treated with SA

(**a**), H_2O_2 (**b**) and HW (**c**) at different exposure times. The values are means of five replicates ± standard deviation (SD).

3.3.5 CMD incidence in sprouted plants from SA, H_2O_2 and HW treated stem cuttings

Sprouted plants from stem cuttings treated with 1% H_2O_2 for 24 h had the highest disease incidence of cassava mosaic disease at 86.7% followed those from stem cuttings treated with 0.5% and 1.5% H_2O_2 for 24 h with 83.3% of plants showing CMD symptoms. Exposure of plants to 1% H_2O_2 for 12 h significantly reduced the disease incidence to 22.2% (Table 3.4). The disease incidence was highest (100%) in plants from stem cuttings treated with 2.5 mM and 5 mM SA for 24 h. The lowest disease incidence (34.9%) was recorded in plants from stem cuttings treated with 5 mM SA for 6 h (Table 3.4). Plants from stem cuttings treated with HW at 55 ºC for 10 and 5 min had the highest disease incidence of 77.7% and 73.2%, respectively. The lowest disease incidence (43.2%) was recorded in sprouted plants from stem cuttings treated with HW at 50 ºC for 5 min (Table 3.4).

Table 3.4 The disease incidence and symptomatology of sprouted plants from stem cuttings treated with H_2O_2, SA and HW at different exposure times.

Time (h)	**H_2O_2 conc. (%)**	**0**	**0.5**	**1**	**1.5**	**Symptoms observed on sprouted plants**
6			56.0 de	56.6 de	79.6 bc	severe/ mild chlorosis, leaf twisting and distorted leaf blades
12			42.59 e	22.2 f	66.7 cd	mild/severe chlorosis, leaf distortion and leaf twisting
24			83.33 b	86.7 ab	83.3 b	severe chlorosis, leaf twisting and distorted leaf blades
Control		100 a				severe chlorosis, leaf twisting and distorted leaf blades
Time (h)	**SA conc. (mM)**	**0**	**1.25**	**2.5**	**5**	
6			82.3 bc	79.4 c	34.9 d	severe/ mild chlorosis, leaf twisting and distorted leaf blades
12			95.24 ab	81.4 bc	84.4 bc	severe/ mild chlorosis, leaf twisting and distorted leaf blades
24			93.3 abc	100 a	100 a	severe chlorosis, leaf twisting and distorted leaf blades
Control		100 a				
Time (min)	**HW Temp. (°C)**	**0**	**50**	**55**		
5			43.2 b	73.2 b		mild/severe chlorosis and leaf twisting
10			47.7 c	77.7 a		severe chlorosis and leaf twisting
Control		100 a				severe chlorosis, leaf twisting and distorted leaf blades

A total of 9 stem cuttings were used for each treatment combination and the experiments were replicated three times. Thus, a total of 27 stem cuttings were used for each treatment combination (concentration/temperature × exposure time). Different lower-case letters within the same column indicate significant differences ($p \leq 0.05$).

3.3.6 Efficiency of virus elimination in plants from stem cuttings treated with H_2O_2, SA and HW and PCR verification of cassava mosaic geminiviruses.

The number of asymptomatic plants after treatment with H_2O_2, SA and HW was recorded to determine the efficiency of the three methods in eliminating CMGs. Treatment of stem cuttings with H_2O_2 at a concentration of 1% for 12 h was the most effective in eliminating CMGs at an efficiency of 77.8% (Table 3.5). Stem cuttings exposed to SA at concentration of 5 mM for 6 h was the most effective in eliminating CMGs at an efficiency of 65.1% (Table 3.5). Hot water treatment of stem cuttings at 50 ºC for 5 min was the most effective in eliminating CMGs with an efficiency of 81.7% (Table 3.5). The percentage of virus-infected plants after all the treatments ranged from 0% to 100% (Table 3.5). However, none of the treatments could completely eradicate cassava mosaic geminiviruses from infected stem cuttings. Elimination of CMD using HW was the most effective treatment.

Table 3.5 Virus elimination efficiencies from sprouted plants after treatments of cassava stem cuttings with H_2O_2, SA and HW.

Time (h)	**H_2O_2 conc. (%)**	**0**	**0.5**	**1**	**1.5**
6			43.9 bc	43.5 bc	20.4 de
12			57.4 b	77.8 a	33.3 cd
24			16.7 e	13.3 ef	16.7 e
Control		0			
Time (h)	**SA conc. (mM)**	**0**	**1.25**	**2.5**	**5**
6			17.7 c	20.6	65.1 a
12			4.8 c	18.7 b	24.4 b
24			6.7 c	0	0
Control		0			
Time (min)	**HW Temp. (°C)**	**0**	**50**	**55**	
5			81.7a	22.9c	
10			31.9b	21.7c	
Control		0			

A total of 9 stem cuttings were used for each treatment combination and the experiments were replicated three times. Thus, a total of 27 stem cuttings were used for each treatment combination (concentration/temperature × exposure time). Different lower-case letters within the same column indicate significant differences ($p \leq 0.05$).

Virus indexing was done for all the sprouted plants that survived after treatment with H_2O_2, SA and HW. The efficiency of elimination of geminiviruses from plants obtained from SA, H_2O_2 and HW treated stem cuttings grown in the glass house for 8 weeks was analyzed using PCR. After stem cuttings treatment with SA, H_2O_2 and HW, all sprouted plants that had no visible symptoms in the glass house tested negative for CMD based on PCR amplification whereas those with symptoms in the glass house tested positive for CMD. Specific PCR products of the positive controls were obtained in all cases, whereas no amplicons were generated in the healthy control plants (Fig. 3.6). The expected amplicon size was 368 bp for ACMV and 650 bp for EACMV.

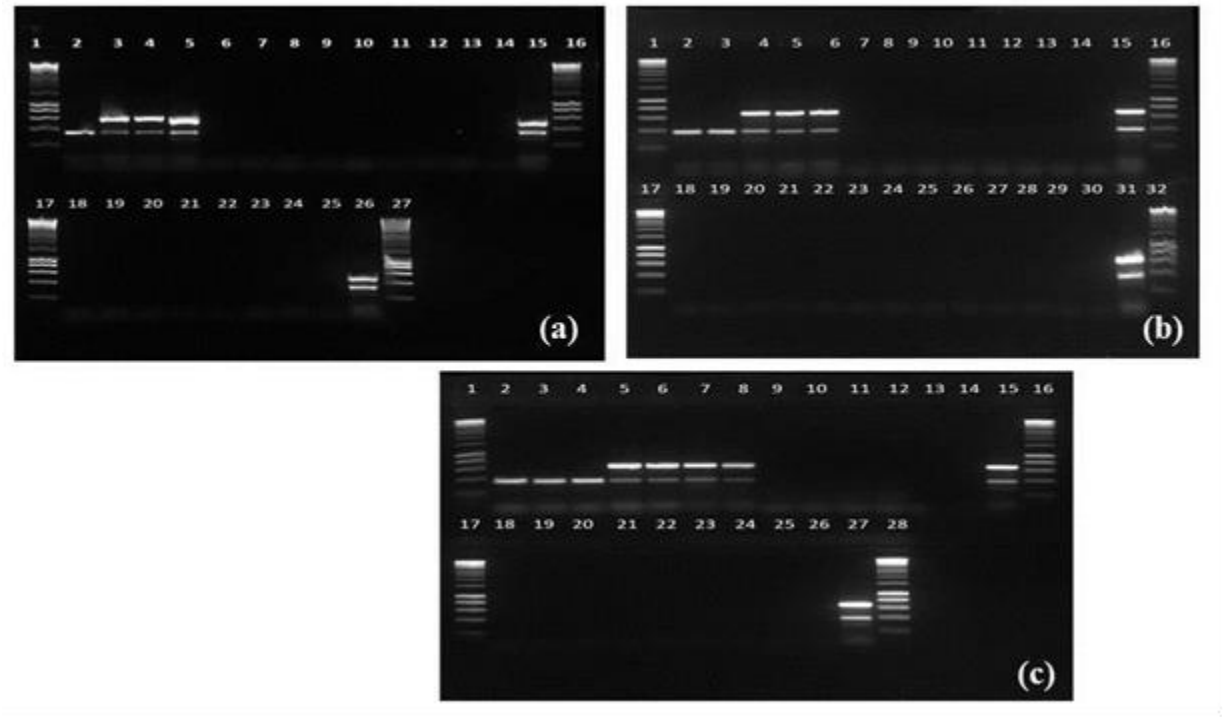


Figure 3.6 Multiplex PCR detection of cassava geminiviruses in sprouted cassava plants after treatment of stem cuttings with H_2O_2, SA and HW.

(a), SA (b) and HW (c) H_2O_2 A: Lanes 1, 16, 17 & 27 = 1kb DNA Hyperladder (Bioline, London, England), Lane 2: = Positive sample for ACMV, Lanes 3, 4 & 5 = samples positive for EACMV, Lanes 7 - 13, and 18 - 24 = samples negative for ACMV & EACMV, Lane 14 & 25 = non-template control, Lane 15 & 26 = Positive control for ACMV & EACMV; B: Lane 1, 16, 17 & 28: DNA ladder (Bioline, London, England), Lanes 2 - 4: Positive sample for ACMV. Lanes 5 - 8: Positive samples for EACMV, Lanes 9 - 13, 18 - 25: samples negative for ACMV & EACMV, Lane 14 & 26: non-template control, Lane 15 & 27; Positive control for ACMV & EACMV; C: Lane 1, 16, 17 & 32: DNA ladder (Bioline, London, England), Lanes 2 & 3: samples positive for ACMV, Lanes 4 - 6: samples positive for EACMV, Lanes 7 - 13, 18 - 29: samples negative for ACMV & EACMV, Lanes 14 & 30: non-template control, Lanes 15 & 31: Positive control for ACMV & EACMV.

3.3.7 Changes in hydrogen peroxide and malondialdehyde levels.

The amount of H_2O_2 and MDA was significantly higher ($P \leq 0.05$) in sprouted plants from the non-treated stem cuttings (controls) compared to stem cuttings that CMGs were successfully eliminated with HW, SA andH_2O_2 treatments (Fig. 3.7). There was no significant difference in the amount of MDA and H_2O_2 in the sprouted plants from treated stem cuttings where CMGs were not eliminated and those from the non-treated control stem cuttings (Fig. 3.7).

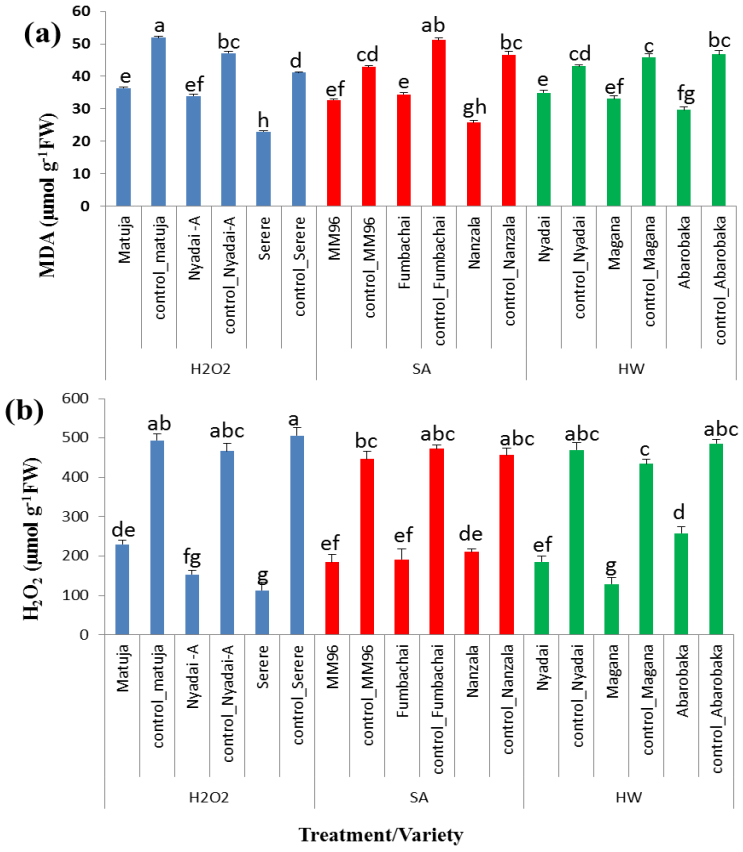


Figure 3.7 The levels of MDA (µmol MDA g-1FW) and H_2O_2 (µmol H_2O_2 g-1FW) in the virus-eliminated plants from SA, H_2O_2 and HW treated stem cuttings and plants from the non-treated control stem cuttings.

The values are means of 5 biological replicates repeated three times (three technical replicates). Different lower-case letters indicate significant differences ($p \leq 0.05$).

3.3.8 Changes in the activities of catalase, peroxidase and ascorbate peroxidase enzymes.

The amount of antioxidant enzymes, CAT, POD and APX was significantly higher ($P \leq 0.05$) in the plants where CMGs were successfully eliminated with HW, SA and H_2O_2 than in the non-treated stem cuttings (Fig. 3.8). There was no significant difference in the amount of CAT, POD and APX in plants where CMG was not eliminated following treatment of stem cuttings with HW, SA and H_2O_2 and in the plants from non-treated control stem cuttings (Fig. 3.8).

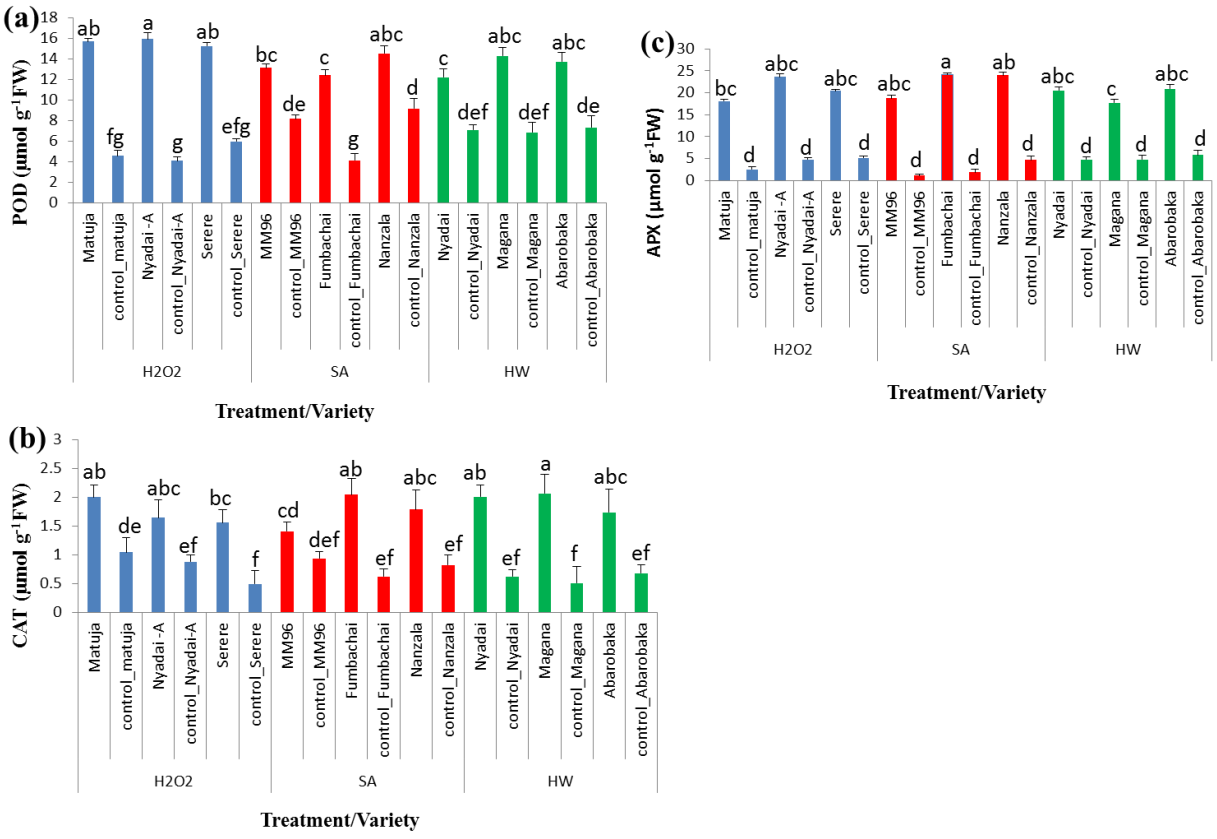


Figure 3.8 The activities of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) (µmol g-1FW) in the virus-eliminated plants obtained from SA, H_2O_2 and HW treated stem cuttings and plants from the non-treated control stem cuttings.

The values are means of three biological replicates repeated three times (three technical replicates). Different lower-case letters indicate significant differences ($p \leq 0.05$).

3.4 Discussion

Cassava is a vegetatively propagated crop and thus the accumulation of viruses over generations reduces the quality and quantity of cassava storage roots (Aimone *et al*., 2021). Currently, viruses can be eliminated from crop plants through different physical and chemical methods to obtain clean healthy planting material. Methods such as tissue culture (meristem culture, somatic embryogenesis), chemical and heat treatments or a combination of tissue culture with chemical and heat treatments have been used to eliminate viruses. Chemical and heat treatments have been reported to be one of the most efficient strategies to control plants diseases through induction of enhanced plant resistance known as systemic acquired resistance (SAR) and is associated with the expression of plant defense genes. Salicylic acid is one of such chemical inducers and plays a key role in plant defense mechanism against pathogens through affecting the physiological and biochemical status of plants such as inducing the expression of pathogenesis related genes and activity of diseases related enzymes (Madhusudhan *et al*., 2009). In this study, exogenous treatments of stem cuttings with 5 mM SA for 6 h were effective in eliminating CMGs from 65.1% of infected stem cuttings. The CMD incidence and severity in plants in form of stem cuttings treated with 5 mM SA for 6 h was lower compared to those from stem cuttings treated with 1.25 mM and 2.5 mM for 6, 12 and 24 h and those treated with 5 mM SA for 12 and 24 h. This result is similar to previous reports by Ong *et al*. (2016), Kidulile *et al*. (2018), Lee *et al*. (2016), and Xi *et al*. (2021) who showed that exogenous application of SA reduced disease incidence and severity in tomato leaves infected with tomato yellow leaf curl virus, in cassava stem cuttings infected with EACMV, and in *Nicotiana benthamiana* infected with tobacco mosaic virus, respectively. Furthermore, Li *et al*. (2019) also reported that exogenous application of SA in tomatoes induced resistance to the geminivirus, tomato yellow leaf curl virus. In this study, growth of sprouted plants

from cassava stem cuttings treated with SA was significantly reduced than those from the non-treated control stem cuttings. The findings concur with those of Mantovani *et al*. (2019) that SA at high concentration can also be toxic to plants and suppresses growth. The results of this study however contradict with the findings of Zhang *et al*. (2019) and Xi *et al*. (2021) whereby SA promoted plant growth rather than suppressing the growth as currently shown in this study. Therefore, it is possible that the effect of SA on growth of plants differs depending on the type of plant.

Hydrogen peroxide (H_2O_2) is also one of the chemical inducers of SAR, which in turn signals the activation of defense genes and formation of phytoalexins (Hernández *et al*., 2016, Liao *et al*., 2013; Kuźniak and Urbanek 2000; Orozco and Ryan, 1999). Hydrogen peroxide is safe and can be detoxified by an antioxidant system in plants. It has been reported that H_2O_2 plays a signaling role in plants under stress and pathogen defense, particularly against virus infection (Radwan and Ismail, 2020). In the current study, treatment of stem cuttings with 1% H_2O_2 eliminated EACMV and ACMV in more than 77.8% of the sprouted plants. Moreover, the treatments lowered the severity of CMD in plants where the virus was not eliminated. These findings are similar to a study by Mejía-Teniente *et al*. (2019), where exogenous application of H_2O_2 protected pepper plants from pepper golden mosaic virus. Radwan and Ismail (2020) found that exogenous application of H_2O_2 in watermelon plants protected the plants from cucumber green mottle mosaic virus infection, demonstrating the useful application of H_2O_2 in a range of plant-virus systems. However, the use of a higher concentration of H_2O_2 in the current study and exposure time compromised the survival rate and growth of plants. This data suggests that H_2O_2 affects cassava survival depending on the dose of H_2O_2 used. Hydrogen peroxide elicits localized cell death in plants and leads to lipid peroxidation that damages the cell membrane, hence the reduction in survival rate in plants

exposed to higher concentrations of H_2O_2 for a longer period (Hernández *et al*., 2016). Other reports have indicated that H_2O_2 enhances plant growth (Nurnaeimah *et al*., 2020). Therefore, it is critical that exposure time and concentration would need to be optimized for a particular cassava variety-virus interaction. Additionally, CMD can be a result of a single geminivirus, such as ACMV, or multiple viruses, including EACMV.

In the present study, it was found that through HW treatment more than 81.7% of virus-free plants could be generated from CMD-infected stem cuttings. Based on the cassava variety used, HW treatment of cassava stem cuttings at 50 ºC for 5 min resulted in the complete eradication of EACMV and ACMV from leaves from 81.7% of sprouted plants. The most efficient HW temperature and exposure time for virus elimination was found to be 50 ºC for 5 min, which eliminated CMGs in 81.7% of the sprouted plants from treated stem cuttings. The high-water temperatures in heat therapy are unfavourable for survival of viruses, and inhibit viral replication (Nangozi *et al*., 2016). While the survival rate of the cassava plants at 50 ºC for 5 min was highest at 77.8% to 100% followed by that of 50 ºC for 10 min at 66.7-88.9%, these conditions may not be suitable for other virus-host systems and need to be optimized. Cassava cuttings subjected to HW treatment at 55 ºC also showed a decrease in disease incidence to 25% in CMD infected plants (Nangozi *et al*., 2016). In a study by Sutrawati *et al*. (2010), pineapple mealy bug wilt-associated DNA viruses were eliminated from pineapple leaves, stems and crown after HW treatment at 58 ºC for 40 min. Higher temperatures destroy plant cells probably due to denaturation of the integral and peripheral proteins making up the cell membrane. Some reports have shown damage to plants due to exposure to high temperatures (Ling, 2010; Damayanti *et al*., 2010), and in these cases this type of treatment may not be applicable to eliminating viruses.

The levels of H_2O_2 and MDA were higher in EACMV and ACMV infected cassava compared to the virus-eliminated cassava plants. Hydrogen peroxide is released in plant cells in high amounts upon abiotic or biotic stress, acting as a signaling molecule to induce pathogen related defense genes. High levels of H_2O_2 can also lead to development of symptoms, notably, chlorosis and mosaic in cassava leaves, similar to CMD (Torres *et al*., 2006). The high levels of H_2O_2 in the cassava leaves of control plants in this study could have led to the development of CMD-type symptoms. On the other hand, suppressed levels of H_2O_2 recorded in the symptom-free plants from the treatments were due to the absence of the virus in the plants as well as overproduction of H_2O_2 detoxifying enzymes CAT, APX and POD. The findings of this study concur with Amoako *et al*. (2015) who reported higher levels of H_2O_2 in ACMV-infected cassava compared with the non-infected cassava plants. Extremely high levels of ROS can be damaging to plants as they may induce cell death by oxidizing proteins, lipids and carbohydrates, among other molecules. This damage to lipids leads to lipid peroxidation which is detectable by measuring the amount of MDA. The results herein are consistent with those of Mishchenko *et al*. (2021), whereby wheat infected with wheat streak mosaic virus had higher levels of H_2O_2 and lipid peroxidation. Lipid peroxidation further enhances the yellowing symptoms observed in viral infected plants due to formation of radical intermediates (Riedle and Bauer, 2000).

In this work, stem cuttings that were exogenously treated with H_2O_2, SA, and HW produced CMD-free plants. It is hypothesized that in plant-virus interactions, the preservation of antioxidant metabolism-related enzymes because of the signaling function of H_2O_2 and SA inhibits viral establishment and disease progression. Salicylic acid has the ability to promote the suppression of the three primary phases of viral infection: reproduction, cell-to-cell transmission, and long-distance transmission (Singh *et al*., 2004). Moreover, it is possible that resistance was created by

a different protective signal transduction route that can be inhibited by salicylhydroxamic acid (Chivasa *et al*., 1997).

The activities of CAT, POD and APX were significantly higher in virus-eliminated cassava plants than in the virus-infected control plants. These are H_2O_2 scavenging enzymes and increasing their activities leads to the formation of lower amounts H_2O_2 (Terzi *et al*., 2018). Catalase works in the first line of defense by breaking down H_2O_2 to water and oxygen and has low affinity for H_2O_2 thus only works efficiently in high concentrations of H_2O_2. APX however is capable of detoxifying even small quantities of H_2O_2 (Asada, 1997; Baker and Orlandi, 1995). Peroxidase is the first enzyme that shows quick defense against viral infection. The enzyme leads to lignification, cell wall elongation, suberization, resistance and polymerization of the cell wall. Enhanced POD activity is correlated with resistance of plants towards phytopathogens (Madhusudhan *et al*., 2009). The antioxidant enzymes are released with an aim of breaking down excessive amounts of H_2O_2 upon pathogen invasion. In this study, the levels of H_2O_2 in the CMD-infected controls remained high due to down-regulation of H_2O_2 detoxifying enzymes, whereas the induction of these antioxidant enzymes in the virus-eliminated plants kept the levels of H_2O_2 low. Similar findings showed a decrease in CAT activity in tomato and bell pepper infected with *tobacco mosaic virus* and *Tomato mosaic virus* compared to the non-infected tomato and bell pepper (Ong *et al*., 2016; Li *et al*. 2019; Xi *et al*., 2021). These plants also showed an accumulation of H_2O_2 and higher levels of lipid peroxidation.

3.5 Conclusion

In conclusion, the findings from this study show that H_2O_2, SA and HW treatments of cassava stem cuttings infected with cassava geminiviruses (EACMV and ACMV) are effective in

eliminating the viruses from planting material. Based on the varieties used in this study, HW treatment at 50 ºC for 5 min and 1% H_2O_2 for 12 h were the most effective in eliminating CMGs with an efficiency of 81.7% and 77.8% respectively. On the other hand, SA eliminated cassava geminiviruses in 65.1% of the infected plant material. The treatments prevented virus replication and halted the progression of the disease by suppressing production of H_2O_2, reducing lipid peroxidation and up regulating the activity of CAT, POD and APX enzymes. Applying and combining the different methods presented in this study and understanding the factors contributing to the success of virus elimination, holds enormous potential to produce virus-free propagation material.

3.6 Recommendations

The effect of variety in response to SA, H_2O_2 and HW treatments was not taken into consideration, and this is a limitation of the current study. The different cassava varieties used in different treatments can influence the outcome of the treatments due to variety × treatment interactions. Different cassava varieties were used for the different treatments, due to the fact that there were not enough stem cuttings of the same variety infected with the target geminiviruses in the field to carry out sufficient experimental replicates. Therefore, from our study, since different varieties were used for different treatments, we were not able to conclude on the best treatment for elimination of geminiviruses from infected stem cuttings. Consequently, the findings from this study focused on determining the effectiveness of the various treatments in eliminating the viruses regardless of the cassava variety used. Therefore, further studies are required to determine the effect of cassava variety in eradication of geminiviruses from infected plants in response to SA, H_2O_2 and HW treatments. There is also need to determine the effect of different treatment combinations such as HW with SA in eradication of geminiviruses from infected plants.

CHAPTER FOUR

4.0 MUTATION INDUCTION USING ETHYL METHYL SULFONATE TO GENERATE RESISTANCE TO CASSAVA GEMINIVIRUSES

Abstract

Cassava mosaic disease (CMD) is a serious constraint to cassava production in sub-Saharan Africa, with the disease causing yield losses of up to 95% in susceptible varieties. Several strategies have been deployed to limit the spread of the disease, but the usage of resistant varieties is the most effective. Chemical-induced mutagenesis has been successfully utilized to generate resistance to diseases in several crop plants. Chemical mutagens are desirable since they result in random point mutations in plants. In the current study, ethyl methyl sulfonate (EMS) was used for induction of mutation in stem cuttings of two CMD-susceptible cassava varieties (Ex-Ndolo and Mucericeri) to enhance resistance to CMD. In addition, two cassava varieties (Karembo and Tajirika) known to be resistant to CMD were also treated with EMS as controls. Seven stem cuttings of each variety were treated with different doses of 0.08% and 0.09% at room temperature of 23 ± 2 ºC for 24 h and the experiment repeated three times. The EMS-treated stem cuttings were washed using tap water to eliminate the EMS adhered to the surface and then planted in plastic pots filled with sterile soil in the glass house. Data on duration of sprouting, sprouting rate, length of stem and number of shoots were recorded after one month. The mutant cassava plants were graft-inoculated with East Africa Cassava Mosaic Virus (EACMV) infected scions. Data on CMD symptom severity score were recorded at 12 and 32 dpi and analyzed by ANOVA. There was a significant decrease in survival rate of stem cuttings and rate of sprouting in the plants for the case of EMS treatments. In addition, there was a significant increase in the stem length and number of shoots for plants from EMS treated stem cuttings. Following graft-inoculation, there was significant decrease in CMD symptom severity and incidence at 32 dpi in putative mutant plants derived from stem cuttings of susceptible cassava varieties Mucericeri and Karembo treated with EMS concentration of 0.09%. Therefore, a dose of 0.09% EMS was significant in reducing CMD symptom severity and incidence in susceptible cassava varieties. The findings from this study lay a foundation for more future research on induced mutagenesis in cassava for inducing geminiviruses resistance.

4.1 Introduction

Although cassava is a crucial crop for food security in developing nations, plant breeding initiatives have frequently overlooked it. Furthermore, traditional breeding initiatives have made a valiant but mostly unsuccessful attempt to overcome many of the obstacles preventing cassava productivity. The difficult breeding technique and its complex genetic makeup have hindered breeding progress. Due to its high heterozygosity, poor flowering, and cross-pollinating nature, cassava is not a good candidate for conventional breeding (Jennings, 2002). Moreover, traditional breeding is usually difficult, labor-intensive, time-consuming, and unsuccessful in producing the desired traits. A promising tactic in view of these is mutation breeding. Induced mutation may offer an alternate strategy for introducing desired traits into current varieties for those features without a source of heritable variation (Jankowicz-Cieslak et al., 2017). Therefore, chemically induced mutation is a possible method for trait improvement in cassava.

Since chemical mutagens introduce genetic variety into higher plants, which is essential for agricultural breeding programs, they are preferred over physical mutagens in terms of their capacity to enhance plant features (Bhat *et al*., 2005). As a result, the mutants make it easier to isolate, identify, and clone the genes needed to create crops with higher yield and higher-quality features (Ahloowalia and Maluszynski, 2001). Reactive oxygen-derived radicals are the primary mechanism by which the majority of chemical mutagens damage plant chromosomes (El-Sayed *et al*., 2012). Following the mutation induction, the harmful effects can occur both artificially and naturally. Large rearrangements brought about by physical mutagens are more harmful than point mutations caused by chemical mutagens. The majority of chemical mutagens result in mutation, which leads to base pair substitution and amino acid exchange, altering the function of proteins without completely destroying them.

Using chemically induced mutagenesis, mutants of a number of crop plants have been created, and they have shown increased disease resistance (Borrelli *et al*., 2018). The most well-known mutations are those that generate resistance to the mildew resistance locus in barley to avoid powdery mildew and those that confer resistance to numerous lettuce diseases in lettuce (Christopoulou *et al*., 2015; Milkis *et al*., 2007). Due to a mildew resistance locus allele found in mutant barley that was generated by deletion, lettuce has maintained resistance against deterioration over the past 20 years. Chemical mutagenesis has been used to boost the resistance of bananas vulnerable to banana bunchy top virus and banana mosaic virus (El-Sayed *et al*., 2012). Cucumber mosaic virus and turnip crinkle virus were partially resistant to *Arabidopsis* due to EMS-induced mutations in cum1 and cum2 (Yoshii *et al*., 2004).

Unlike genetic engineering methods, mutation breeding has been the most successful in producing hundreds of elite mutant varieties in various crop plants with the least amount of ethical and social criticism. In the International Atomic Energy Agency (IAEA) database, 3,402 mutant varieties have been identified; however, only two of the 3402 mutations are related to cassava (Forster and Shu, 2012). The two mutants, Tekbankye and Fuxuan, were produced by gamma radiation and released in Ghana and China, respectively, in 1997 and 2005 (Asare and Safo-Kantanka, 1995).

This data indicates that cassava is a neglected crop in induced mutagenesis. Therefore, it is important to develop cassava mutants with preferred traits such as CMD resistance in farmer-preferred susceptible germplasm through induced chemical mutagenesis.

In the present study, chemical mutagenesis was induced in farmer-preferred CMD-susceptible cassava germplasm by employing ethyl methyl sulfonate (EMS) as a mutagen and the resultant putative mutant plants were screened for resistance to CMD. The strong alkylating agent EMS is

known to change nucleotides via mismatch matching with thymine rather than cytosine, changing G/C pairing to A/T pairing in subsequent DNA repair (Verma *et al*., 2012). The study was conducted to hypothesize the mutagenic effects of EMS on farmer-preferred CMD susceptible cassava varieties.

4.2 Materials and methods

4.2.1 Cassava materials and virus indexing of the plants

Farmer-preferred varieties collected from farmer's fields and confirmed to be susceptible to CMD in Chapter 2 were used for generation of putative mutants for CMD resistance. The susceptible varieties used were Ex-Ndolo and Mucericeri with CMD severity score of 4 and 5, respectively. CMD-resistant cassava varieties Tajirika and Karembo with CMD severity score of 1 were used as controls. Before treatments with the chemical mutagen, the selected cassava varieties were confirmed to be virus-free by PCR using geminiviruses specific primers described in chapter 2.

Genomic DNA was isolated from young leaves from stems intended for chemical mutagen treatments. The extraction process was followed exactly as Osena *et al*. (2017) explained. A total of 12.5 µl of Quick-Load Taq 2× master mix (New Englands Biolabs), 0.5 µl of 10 µM of each of the forward and reverse primers (explained in chapter 2), 2 µl of DNA template, and 9.5 µl of nuclease-free PCR-grade water were used to prepare the PCR reaction in a final reaction volume of 25 µl.

The PCR was performed using an Eppendorf AG 22331, Hamburg, Germany, conventional thermocycler. The temperature profile for the PCR was as follows: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing for both primers at 55 °C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. Gel electrophoresis in 1.5%

w/v agarose was run on amplified products. The experiment involved conducting electrophoresis in 0.5 × TBE buffer and viewing the fragments using GelRed dye (Biotium, Fremont, CA, USA) under an Azure C200 transilluminator (Biosystems, USA). A 1kb ladder (Bioline, London, England) was used to measure the sizes of the DNA fragment.

4.2.2. Treatment of cassava stem cuttings with EMS.

The EMS solution was prepared at a concentration of 0.08% and 0.09%. Seven cassava stem cuttings of each variety were used for each treatment and the experiment repeated three times.

4.2.2.1 Preparation of EMS solution

The EMS preparation process was completed in accordance with Mba *et al*. (2010). Using distilled water and dimethyl sulfoxide (DMSO) as the carrier agent, a fresh EMS solution was made. The necessary amounts of water were mixed with 5% (v/v) DMSO, autoclaved for 15 minutes at 15 psi at 120 °C, and then allowed to cool at room temperature. In order to create a homogeneous mixture, EMS mutagen was added to the sterilized water-DMSO mixture in a laminar flow cabinet at concentrations of 0.08% and 0.09% and vigorously shaken. Starting with the lowest concentrations, the needed volumes of EMS solution were measured using a sterile syringe and a 0.2 µl filter in the sterile water-DMSO mixture.

A decontaminating solution containing 10% (w/v) sodium thiosulphate was also prepared. EMS in water-DMSO mixture with concentrations of 0.08% and 0.09% as well as water-DMSO without EMS as control was used for the treatment of stem cuttings.

4.2.2.2 Treatment of cassava stem cuttings using EMS solution and disposal of the EMS after treatments.

Equal volumes of the EMS-water-DMSO mixture were added to each container labeled with the cassava variety, EMS concentration, and incubation duration.

For each cassava variety, seven stem cuttings (10 cm in length with three nodes) were immersed in 0%, 0.08% and 0.09% EMS for 24 h as described by Baguma *et al*. (2021) and the experiment was repeated three times. The treatments were kept at room temperature at 23 ± 2 ºC under the fume chamber. The controls were treated with sterilized water-DMSO mixture without EMS.

The EMS solution was properly decanted and the stem cuttings were extensively rinsed (three to five times) with tap water following their 24-hour incubation. After detoxifying by adding 10% (w/v) sodium thiosulfate in a 3:1 volume ratio and letting it stand for 30 hours, the leftover EMS solution and wash water were combined and placed in a hazardous waste container for disposal. Sodium thiosulfate was used to disinfect the work environment, and all equipment contaminated by EMS were disposed off in compliance with safety regulations.

4.2.3 Establishment of EMS-treated cuttings in plastic pots in the glass house and collection of morphological data

The EMS-treated stem cuttings (one stem per pot) were planted in plastic pots (Kenpoly, Kenya) containing sterilized farmyard manure and sterilized soil at a 1:3 (v/v) ratio. The pots received twice weekly irrigation after being irrigated to field capacity once a day till they sprouted. The sprouted plants were kept in a controlled glasshouse at a temperature of 26/16±2 ºC (day/night), 70% relative humidity, and a photoperiod of 16/8 hours (day and night).

Data on sprouting rate (the number of days taken for emergence of the first shoot) were recorded weekly after EMS treatment for six weeks when the plants were ready for grafting. Data on growth parameters (number of shoots and length of stems) and survival rate were also recorded for six weeks. Stem length was measured using a 30 cm ruler, from the base of the plant to the last stem node.

4.2.4 Screening of cassava mutants for CMD resistance in the glasshouse.

The putative mutant plants were screened for resistance to CMD by graft-inoculation with EACMV.

4.2.4.1 Infection of putative mutant plants with East Africa cassava mosaic virus and CMD symptom severity score.

Putative mutant plants were screened for resistance to CMD. The putative mutants were inoculated with EACMV using grafting. The source of the inoculum was from CMD-susceptible cassava variety MM96/4884 with a severity score of 5 exhibiting total distortion of 4/5 of the leaves as described by Houngue *et al*. (2019). The inoculum source plants were confirmed to be positive for EACMV and negative for ACMV through PCR as described by Alabi *et al*. (2008). The plants were collected from farmers' fields and established in a separate greenhouse to serve as a source of EACMV inoculum.

Six weeks-old putative mutant plants of the four cassava varieties were inoculated with EACMV using grafting method (Anjanappa *et al*., 2016). The inoculum was grafted onto the test plants to enable the transfer of the viral particles, with the putative mutant plants of the four cassava varieties acting as the rootstock (Anjanappa *et al*., 2016). A sterile blade was used to cut a vertical incision on the stem of putative mutant plants. The phloem and xylem of the two plants corresponded when

a scion of the inoculum plants was cut and placed in the slit of the presumed mutant plants. In order to enable union, graft sections were firmly taped using parafilm. To protect them from excessive evaporation, grafted plants were covered with plastic bags.

After 7 days the polythene bags were removed. Control plants were also graft-inoculated with diseased scions of cassava variety MM96/4884. Each cultivar consisted of seven pots (five biological replicates) and repeated three times. All the plants were kept in an insect-proof glasshouse at a temperature of 26/16 ± 2 ºC (day/night) and 70% relative humidity. The graft-inoculated putative mutants were monitored weekly for CMD symptoms expression and symptom severity scores were recorded over a period of 32 days.

Leaves below the apex for EACMV- and mock-inoculated plants were sampled at time-points 12 and 32-dpi (Allie *et al*., 2014). Leaves were collected from seven biological replicates for each variety, each treatment (EACMV- and mock-inoculated) and at three time points. Each biological replicate was tested in three independent technical replicates. Leaves from the different time points were sampled, immediately frozen in liquid nitrogen and stored at -80 ºC until subsequent use for DNA extractions.

4.2.4.2 Detection of EACMV in graft-inoculated putative mutant plants

Detection of the EACMV in the inoculated putative mutant plants was done by PCR analysis. Genomic DNA was isolated from young leaves following the extraction protocol outlined by Osena *et al*. (2017). The PCR reaction was prepared using 12.5 µl of Quick-Load Taq 2× master mix (New Englands Biolabs), 0.5 µl of 10 µM of each forward and reverse primer (described in chapter 2), 2 µl of DNA template, and 9.5 µl of molecular grade water, resulting in a final reaction volume of 25 µl. The PCR was conducted using an Eppendorf AG 22331, Hamburg, Germany,

conventional thermocycler, with the following temperature profile: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing for both primers at 55 °C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. Subsequently, 1.5% w/v agarose gel electrophoresis was carried out to analyze the amplified products. The 0.5 × TBE buffer was used for the electrophoresis, and a transilluminator (Azure C200, Biosystems, USA) was used to visualize the fragments using GelRed stain (Biotium, Fremont, CA, USA). A 1 kb ladder (Bioline, London, England) was used to measure the size of the fragments.

4.2.5 Data analysis

The data on growth parameters (stem length and number of shoots), survival percentage and number of days after first sprouting and virus score were recorded in excel sheets and the data subjected to two-way ANOVA using Genstat version 15 software. Separation of means was done using Tukey HSD test at 5% probability level. Graphs and tables with the means were generated using the Genstat software.

4.3 Results

4.3.1 Effects of EMS on the duration of sprouting of cassava stem cuttings.

There was a significant difference ($P \leq 0.05$) in the time of sprouting of the cuttings treated with EMS compared to the non-treated controls among the cassava varieties. The EMS- treated stem cuttings took significantly longer time to sprout compared to the non-treated controls (Table 4.1). The duration of sprouting of stem cuttings was affected at higher dose of EMS. For all the tested cassava varieties except Karembo, stem cuttings treated with 0.09% EMS took a significant longer time to sprout compared to the stems treated with 0.08% EMS. The results on the duration of sprouting of stem cuttings negatively correlate with the EMS doses compared with the control.

Table 4.1 Effect of EMS on the duration of sprouting of plants

EMS concentration (%)	Days taken to sprout			
	Ex-Ndolo	**Karembo**	**Mucericeri**	**Tajirika**
0	11.67 d	11.36 d	7.89 e	11.14 d
0.08	14.33 c	11.08 d	12.53 d	11.39 d
0.09	17.64 b	11.88 d	14.48 c	19.77 a

Seven stem cuttings were used per treatment and the experiment was repeated three times. Different lower-case letters within the same column indicate significant difference ($p \leq 0.05$)

4.3.2 Effect of EMS on the survival/sprouting rate of plants.

The sprouting/survival rate of EMS-treated stem cuttings was significantly reduced ($p \leq 0.05$) compared to the non-treated controls. The least survival percentage was recorded in plants of stem cuttings of 0.09% EMS treated varieties Karembo and 0.08% EMS treated variety Mucericeri followed by 0.08% EMS treated Tajirika and Ex-Ndolo. However, the survival percentage of variety Mucericeri treated with 0.8% EMS remained at 100%. All non-treated control plants of each variety recorded 100% survival percentage (Figure 4.1).

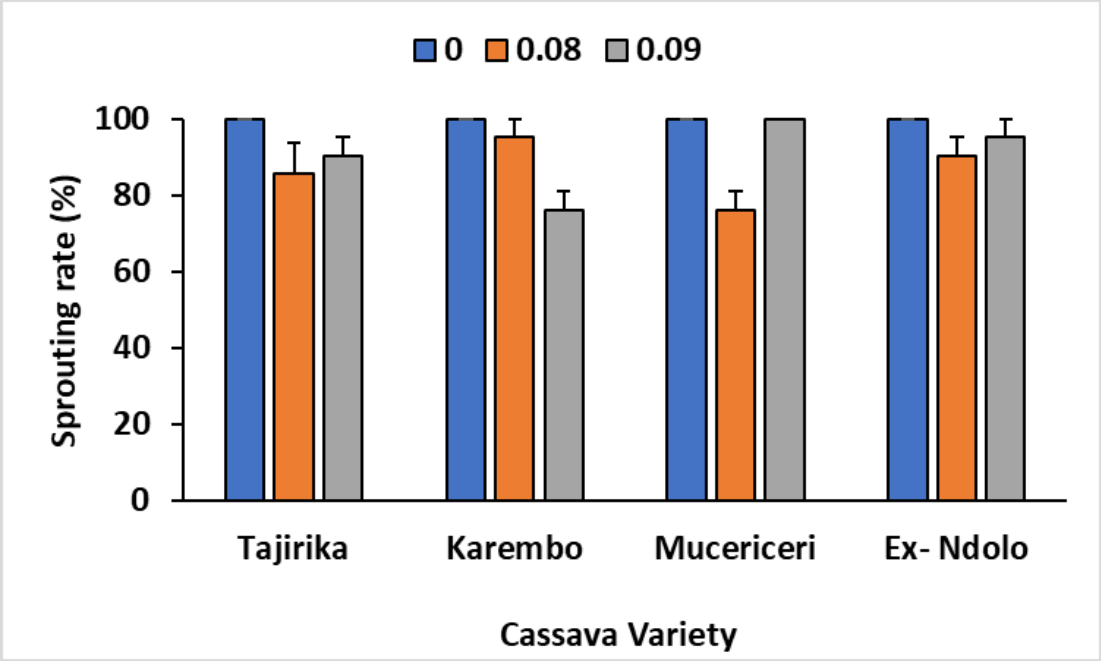


Figure 4.1: The effect of EMS on percentage survival of plants of treated stem cuttings.

The data was collected for the 21 plants (seven experimental plants done in triplicates) and was collected on week six after establishment of the treated cuttings in the glass house

4.3.3 Growth rate and morphological analysis of putative mutant plants derived from EMS-treated stem cuttings.

Treatment of cassava stem cuttings with EMS affected the growth and morphology of putative mutant plants. There was a significant difference ($p \leq 0.05$) in the number of shoots and the length of stems in plants derived from EMS-treated stem cuttings compared to the non-treated controls in all the varieties. Stem cuttings treated with EMS produced significantly high number shoots compared to the plants derived from non-treated stem cuttings. Similarly, the length of stems in plants derived from EMS-treated stem cuttings were significantly longer (Figure 4.2) compared to stems of plants derived from non-treated stem cuttings (Table 4.2).

Putative mutants Ex-Ndolo and Mucericeri (susceptible varieties) recorded a significantly high number of shoots for putative mutant plants derived from 0.08% and 0.09% EMS treated stem cuttings. Mutant plants of variety Karembo had the lowest number of shoots in both concentrations of EMS when compared to the putative mutants derived from other varieties. Putative mutant plants of variety Karembo had the most significant increase in stem length at both EMS concentrations followed by Ex-Ndolo and Mucericeri whereas Tajirika had the most significant reduction in stem length at both concentrations of EMS (Table 4.2). Generally, the number of shoots and length of stems in putative mutant plants exhibited a reduction at higher dose of EMS.

Table 4.2 Growth parameters of putative mutant plants derived from EMS treated and non-treated control stem cuttings

EMS concentration (%)	No. of shoots				Stem length (cm)			
	Ex-Ndolo	Karembo	Mucericeri	Tajirika	Ex-Ndolo	Karembo	Mucericeri	Tajirika
0	5.1 b ±0.33	4.61 c± 0.11	4.64 c±0.39	3.95 d±0.12	7.94f±0.79	13.1b±1.61	7.58f±0.89	5.48g±0.53
0.08	6.45 a±0.2	5.36 b±0.19	5.95 b±0.13	5.58 b±0.28	11.32c±1.19	14.25a±1.36	10.63c±1.63	7.68f±1.35
0.09	5.83 a ±1.1	5.11 b±0.3	5.18 b±0.74	4.26 c±0.56	9.35d±2.06	13.78a±1.00	8.63e±1.49	7.37f±0.71

Seven stem cuttings were used per treatment and the experiment was repeated three times. Different lower-case letters within the same column indicate significant difference ($p \leq 0.05$)

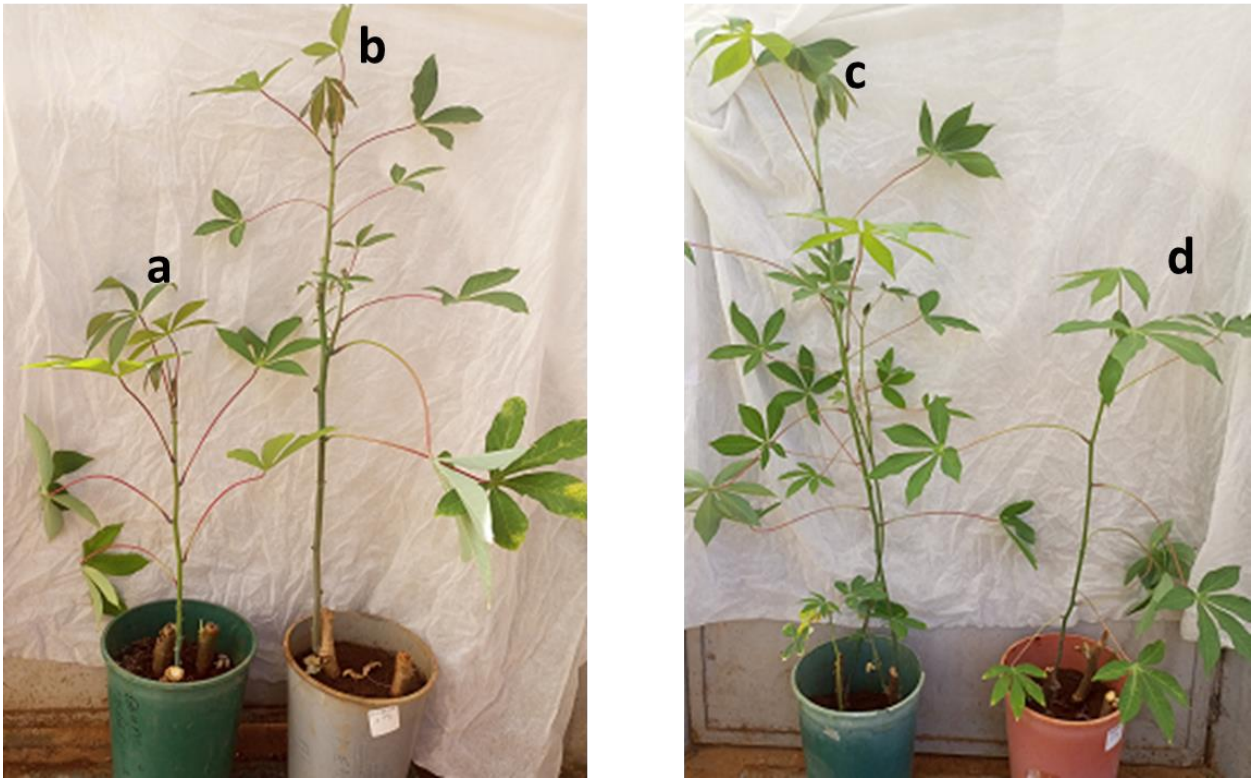


Figure 4.2 Putative mutant plants of EMS treated stem cuttings showing increase in length of stems

(a) non-treated control plant of variety Karembo, (b) EMS treated variety Karembo, (c) EMS treated variety Ex-Ndolo and (d) non-treated control plant Ex-Ndolo.

There were morphological changes in putative mutant plants of variety Tajirika derived from 0.09% EMS treated stem cuttings. The mutant plants exhibited large and broad leaves compared to the control plants derived from non-treated stem cuttings (Figure 4.2). The leaf surface area of three putative mutant plants of variety Tajirika derived from stem cuttings treated with 0.09% EMS was 72 cm^2, 74.4 cm^2 and 69.6 cm^2 whereas the plants derived non-treated control stem cuttings recorded leaf surface area of 32 cm^2 (Table 4.3). The rest of the putative mutant varieties did not exhibit any significant difference ($P \leq 0.05$) in their leaf surface area compared to their control.

Leaves of putative mutant plants of variety Karembo derived from stem cuttings treated with 0.09% EMS exhibited a glossier appearance compared to the plants derived from non-treated stem cuttings (Figure 4.4). Putative mutant plants of varieties Mucericeri, Tajirika and Ex-Ndolo derived from EMS treated stem cuttings did not exhibit any changes in cuticular wax in comparison with their respective control plants.

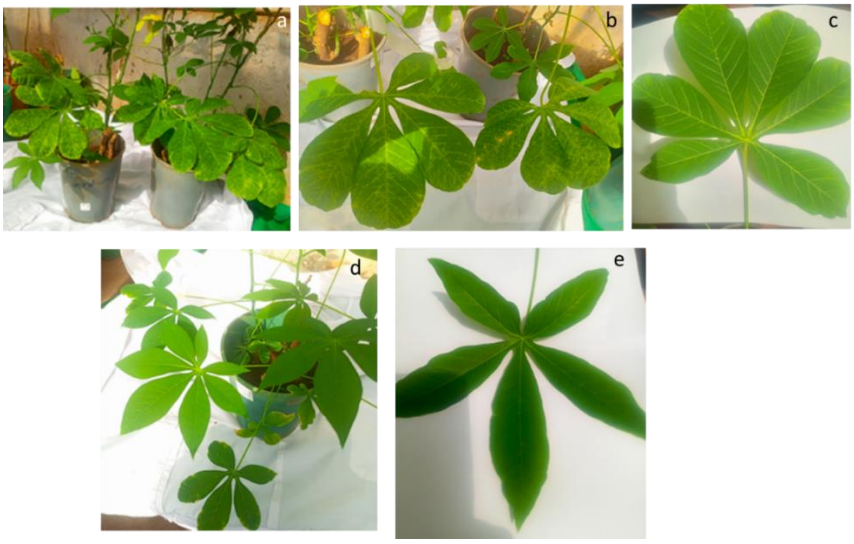


Figure 4.3: Morphological variations of leaves from putative mutant plants of EMS-treated stem cuttings of variety Tajirika

(**a**), (**b**) and (**c**) Broad leaves of plants from stem cuttings treated with 0.09% EMS; and (**d**) and (**e**) normal leaves of control plants from non-treated stem cuttings.

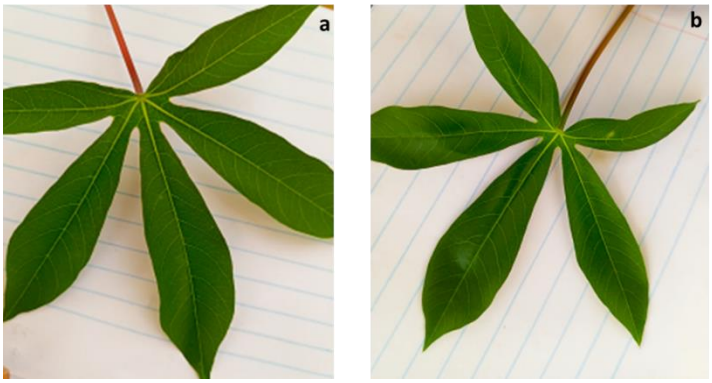


Figure 4.4 Variations in the cuticle of EMS treated and non-treated variety Karembo.

(**a**) Leaf from control plant of variety Karembo; (**b**) glossy appearance of leaf from putative mutant plant of variety Karembo after treatment of stem cuttings with 0.09% EMS.

Table 4.3 Leaf surface area of putative mutant plants after treatment with EMS

Cassava variety	EMS treatment (%)	Putative mutant	Leaf surface area (cm^2)
Ex-Ndolo	0.09	Mutant #1	36
		Mutant #2	28
		Mutant #3	30
	0.08	Mutant #1	31.2
		Mutant #2	23
		Mutant #3	29.64
	0	control	21.6
Mucericeri	0.09	Mutant #1	42
		Mutant #2	40.5
		Mutant #3	42.92
	0.08	Mutant #1	46.8
		Mutant #2	54.6
		Mutant #3	56
		control	56
Tajirika	0.09	Mutant #1	72
		Mutant #2	74.4
		Mutant #3	69.6
	0.08	Mutant #1	32
		Mutant #2	38.4
		Mutant #3	32.8
		control	32
Karembo	0.09	Mutant #1	27
		Mutant #2	28
		Mutant #3	31.2
	0.08	Mutant #1	29
		Mutant #2	23.6
		Mutant #3	27
		control	24

4.3.4 Response of putative mutant plants to EACMV infection.

The response of the EACMV-inoculated putative mutant plants was evaluated by observing the symptoms, recording the CMD symptom severity score and disease incidence. Detection of EACMV in inoculated plants was done by PCR using EACMV-specific primers.

4.3.4.1 Symptomatology of putative mutant plants after EACMV inoculation.

The EMS treated varieties exhibited no symptoms at 12 dpi across all concentrations and in all controls except in control variety Mucericeri that exhibited mild CMD symptoms.

At 32 dpi, all varieties treated with 0.08% EMS and their controls exhibited CMD symptoms except EMS-treated variety Tajirika and the control that were asymptomatic. At 0.09% EMS concentration, all cassava varieties exhibited no CMD symptoms.



Figure 4.5 Symptoms of putative mutant plants from EMS-treated stem cuttings of variety Tajirika after inoculation with EACMV at 12 dpi

(a) Asymptomatic variety Tajirika treated with 0.08% EMS, (b) Asymptomatic variety Tajirika treated with 0.09% EMS and (c) Asymptomatic control variety Tajirika

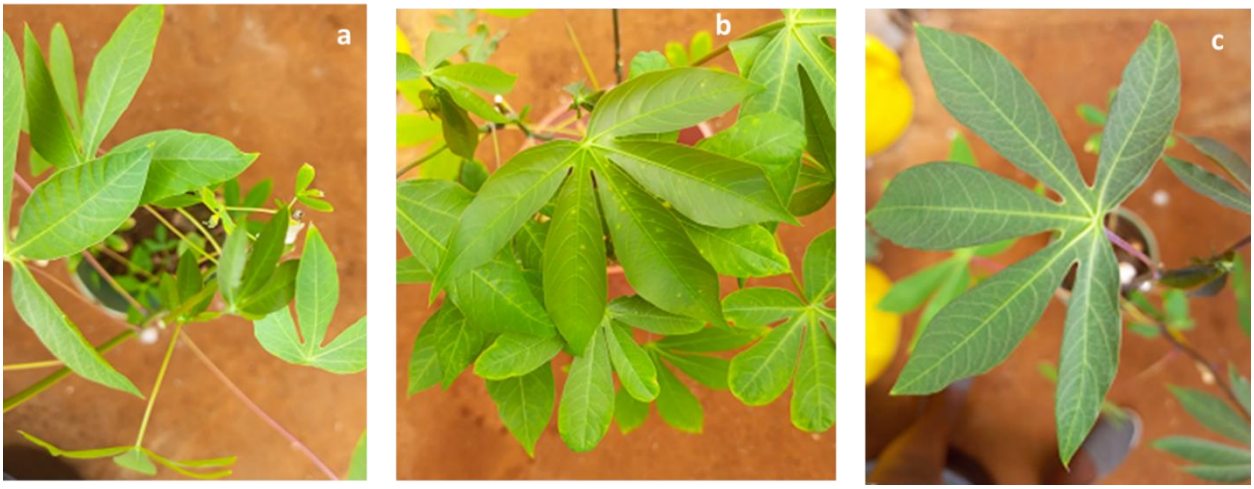


Figure 4.6 Symptoms of putative mutant plants from EMS-treated stem cuttings of variety Karembo after inoculation with EACMV at 12 dpi

(a) Asymptomatic variety Karembo treated with 0.08% EMS, (b) Asymptomatic variety Karembo treated with 0.09% EMS and (c) Asymptomatic control variety Karembo

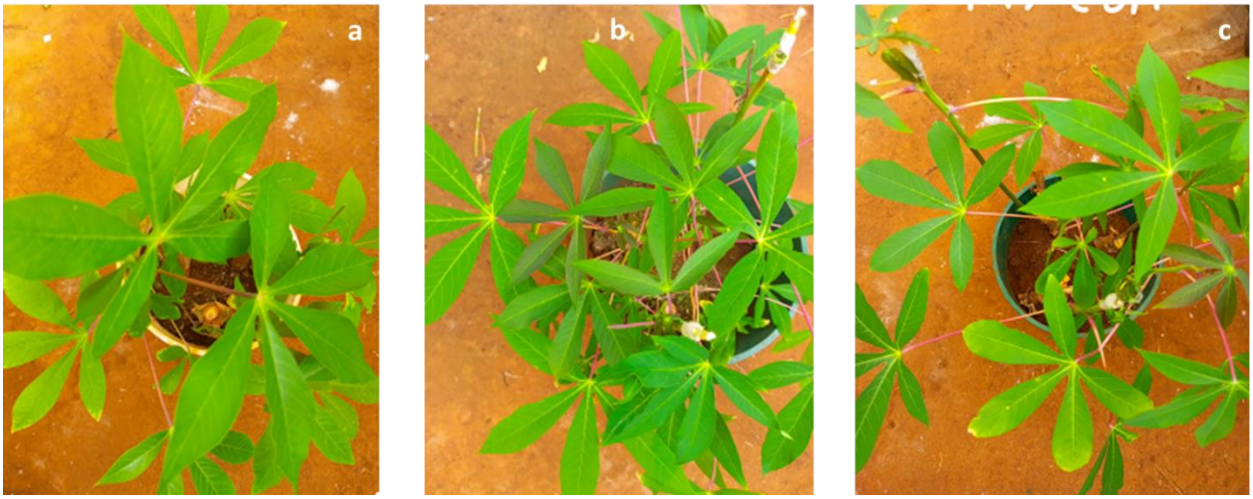


Figure 4.7 Putative mutant plants from EMS-treated stem cuttings of variety Ex-Ndolo after inoculation with EACMV at 12 dpi

(a) Asymptomatic variety Ex-Ndolo treated with 0.08% EMS, (b) Asymptomatic variety Ex-Ndolo treated with 0.09% EMS and (c) Asymptomatic control variety Ex-Ndolo



Figure 4.8 putative mutant plants from EMS-treated stem cuttings of variety Mucericeri after inoculation with EACMV at 12 dpi

(a) Asymptomatic variety Mucericeri treated with 0.08% EMS, (b) Asymptomatic variety Mucericeri treated with 0.09% EMS and (c) Symptomatic control variety Mucericeri



Figure 4.9 Symptoms of putative mutant plants from EMS-treated stem cuttings of variety Karembo after inoculation with EACMV at 32 dpi

(a) Symptomatic variety Karembo treated with 0.08% EMS, (b) Asymptomatic variety Karembo treated with 0.09% EMS and (c) Symptomatic control variety Karembo



Figure 4.10 Symptoms of putative mutant plant from EMS-treated stem cuttings of variety Ex-Ndolo after inoculation with EACMV at 32 dpi

(a) Symptomatic variety Ex-Ndolo treated with 0.08% EMS, (b) Asymptomatic variety Ex-Ndolo treated with 0.09% EMS and (c) Symptomatic control variety Ex-Ndolo

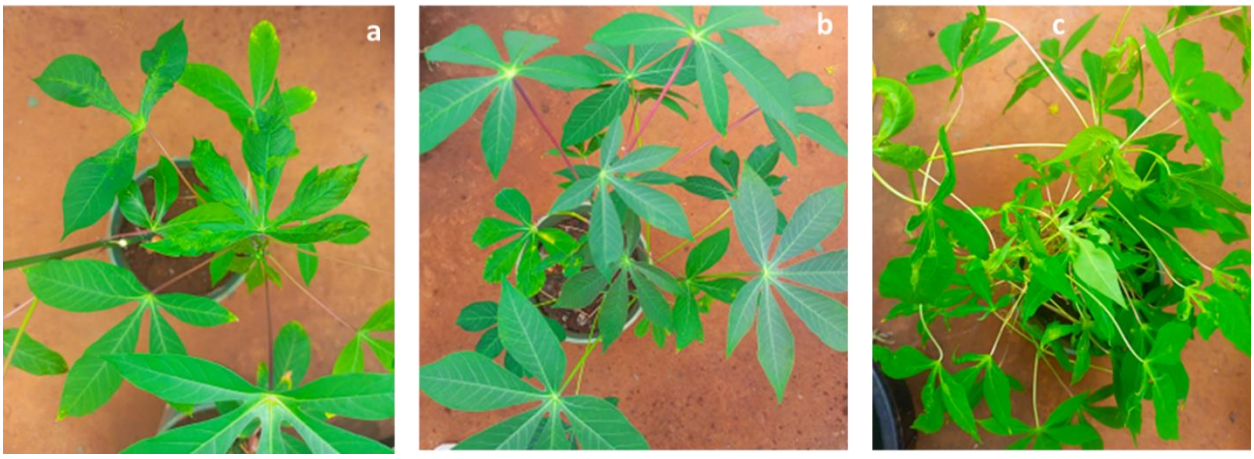


Figure 4.11 Symptoms of putative mutant plant from EMS-treated stem cuttings of variety Mucericeri after inoculation with EACMV at 32 dpi

(a) Symptomatic variety Mucericeri treated with 0.08% EMS, (b) Asymptomatic variety Mucericeri treated with 0.09% EMS and (c) Symptomatic control variety Mucericeri

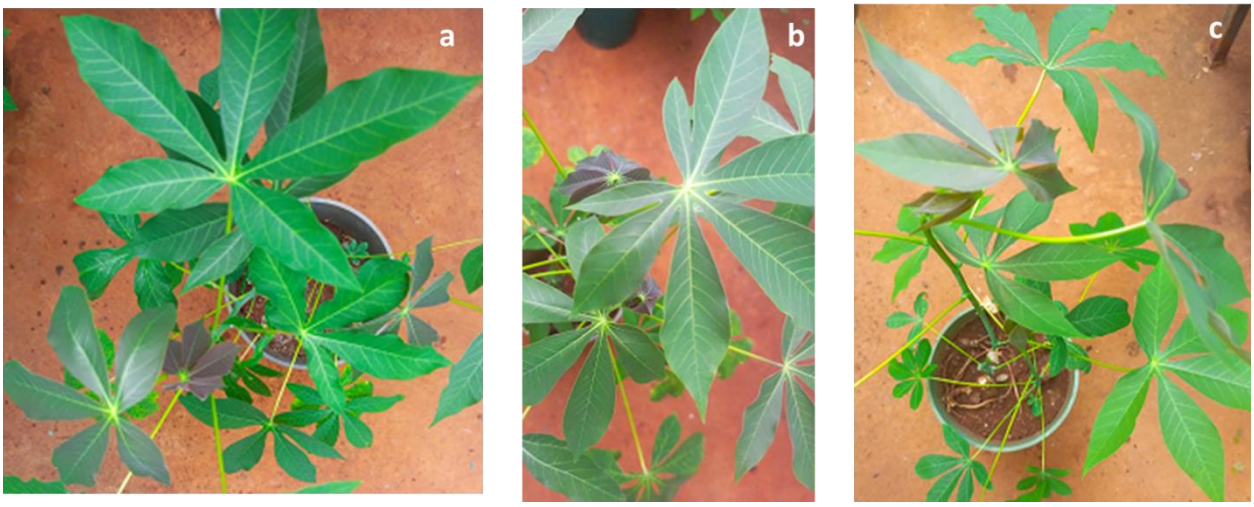


Figure 4.12 Symptoms of putative mutant plant from EMS-treated stem cuttings of variety Tajirika after inoculation with EACMV at 32 dpi

(a) Asymptomatic variety Tajirika treated with 0.08% EMS, (b) Asymptomatic variety Tajirika treated with 0.09% EMS and (c) Asymptomatic control variety Tajirika

4.3.4.2 CMD disease incidence of putative mutant plants derived from EMS-treated cassava stem cuttings

There was no disease incidence in all putative mutant plants derived from0.08%, and 0.09% EMS treated stem cuttings for all cassava varieties at 12 dpi (Figure 4.13 a). The non-control plants of all cassava varieties also recorded no disease incidence at 12 dpi except for plants of variety Mucericeri that had an incidence of 60%.

At 32 dpi, the disease incidence was highest (100%) in putative mutant plants of varieties Mucericeri and Ex-ndolo treated with 0.08% EMS followed by Karembo (20%) (Figure 4.13 b). Putative mutant plants derived from 0.08% EMS treated stem cuttings of variety Tajirika recorded no disease incidence. Non-treated control plants of Mucericeri and Ex-Ndolo had a disease incidence of 100%, whereas the non-treated controls of Karembo and Tajirika had zero disease incidences (Figure 4.13 b).

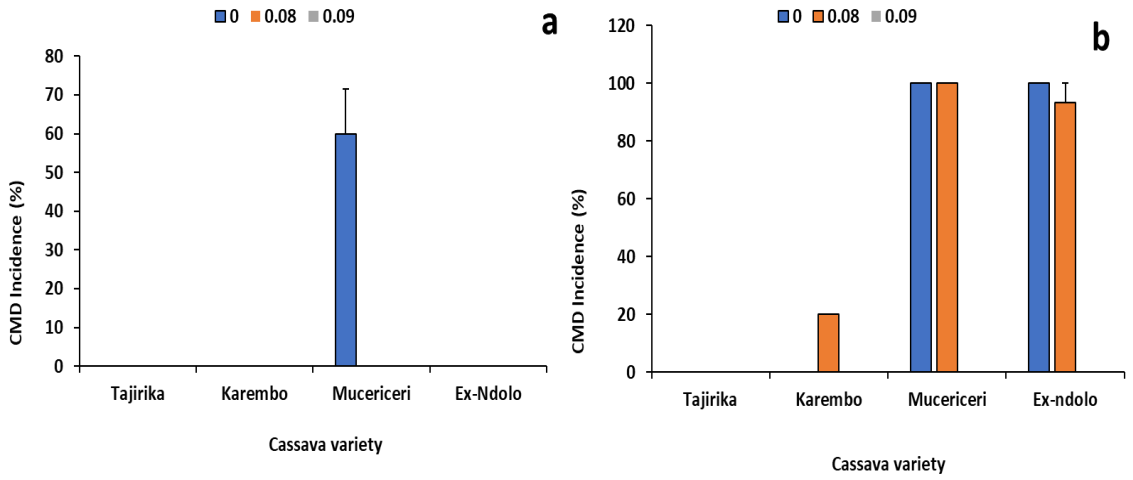


Figure 4.13 Cassava mosaic disease incidences of putative mutant plants derived from stem cuttings treated with 0.08% and 0.09% EMS at 12 dpi (a) and at 32 dpi (b).

4.3.4.3 CMD severity of the putative mutant plants after inoculation with EACMV

At 12 dpi, CMD severity score of 1 was obtained for all putative mutant plants derived from EMS treated cassava stem cuttings and control plants from the non-treated stakes except in the non-treated control plants of variety Mucericeri that recorded a CMD severity score of 2 (Table 4.4). At 32 dpi, variety Mucericeri stem cuttings treated with 0.08% EMS had the highest number of putative mutant plants displaying typical CMD symptoms, with five out of seven mutant plants having a CMD severity score of 2 and two having a score of 3. Variety Ex-Ndolo stem cuttings treated with 0.08% EMS at 32 dpi had six out of seven of the putative mutants recording a severity score of 2 and the remaining one having a CMD severity score of 1. Karembo treated with 0.08% EMS at 32 dpi had three out of seven of the putative mutant plants having a CMD severity of 2 whereas the rest had a score of 1 (Table 4.4). All putative mutants of variety Tajirika recorded a severity score of 1 at both 0.08% EMS and 0.09% EMS treatments. At 32 dpi, control plants of varieties Mucericeri and Ex-Ndolo (susceptible varieties) recorded the highest disease severity

score, whereas controls of Karembo and Tajirika (resistant varieties) had a severity score of 1 (Table 4.4). At 0.09% EMS concentration, the severity score remained at 1 in all the putative mutant plants of EMS treated stem cuttings for all the cassava varieties tested (Table 4.4).

Table 4.4 CMD severities of putative mutants derived from stem cuttings treated with different concentrations of EMS at 12 dpi and 32 dpi.

Variety	EMS (%)	Putative mutant	CMD symptom severity score	
			12 dpi	32 dpi
Tajirika	0.08	Mutant #1	1	1
		Mutant #2	1	1
		Mutant #3	1	1
		Mutant #4	1	1
		Mutant #5	1	1
		Mutant #6	1	1
		Mutant #7	1	1
	0.09	Mutant #1	1	1
		Mutant #2	1	1
		Mutant #3	1	1
		Mutant #4	1	1
		Mutant #5	1	1
		Mutant #6	1	1
		Mutant #7	1	1
	0	Control	1	1
Karembo	0.08	Mutant #1	1	2
		Mutant #2	1	2
		Mutant #3	1	2
		Mutant #4	1	1
		Mutant #5	1	1
		Mutant #6	1	1
		Mutant #7	1	1
	0.09	Mutant #1	1	1
		Mutant #2	1	1
		Mutant #3	1	1
		Mutant #4	1	1
		Mutant #5	1	1

		Mutant #6	1	1
		Mutant #7	1	1
	0	Control	1	1
Mucericeri	0.08	Mutant #1	1	2
		Mutant #2	1	2
		Mutant #3	1	2
		Mutant #4	1	2
		Mutant #5	1	2
		Mutant #6	1	3
		Mutant #7	1	3
	0.09	Mutant #1	1	1
		Mutant #2	1	1
		Mutant #3	1	1
		Mutant #4	1	1
		Mutant #5	1	1
		Mutant #6	1	1
		Mutant #7	1	1
	0	Control	2	3
Ex-Ndolo	0.08	Mutant #1	1	1
		Mutant #2	1	2
		Mutant #3	1	2
		Mutant #4	1	2
		Mutant #5	1	2
		Mutant #6	1	2
		Mutant #7	1	2
	0.09	Mutant #1	1	1
		Mutant #2	1	1
		Mutant #3	1	1
		Mutant #4	1	1
		Mutant #5	1	1
		Mutant #6	1	1
		Mutant #7	1	1
	0	Control	1	3

Tajirika and Karembo are varieties resistant to CMD; Ex-Ndolo and Mucericeri are susceptible to CMD.

4.3.4.4 Detection of EACMV in inoculated putative mutant plants

The stem cuttings of the tested cassava varieties were confirmed to be EACMV-free before treatment with EMS (Figure 4.13). The putative mutant plants were graft-inoculated with EACMV and tested for the presence of EACMV at 12- and 32-dpi. Putative mutant plants derived from stem cuttings varieties Karembo, Tajirika, Mucericeri and Ex-Ndolo treated with 0.08% and 0.09% EMS tested negative for EACMV using PCR at 12 dpi. The control plants of varieties Karembo, Tajirika and Ex-Ndolo also tested negative for EACMV using PCR at 12 dpi. The control plants of variety Mucericeri tested positive for EACMV using PCR at 12 dpi (Figure 4.14). At 32 dpi, 7 putative mutants of varieties Mucericeri, 6 of variety Ex-Ndolo and 3 of variety Karembo treated with 0.08% EMS and their controls tested positive for EACMV using PCR (Table 4.5). Tajirika treated with 0.08%, 0.09% and the control tested negative for EACMV. Putative mutants of Mucericeri, Ex-Ndolo and Karembo treated with 0.09% EMS tested negative for EACMV using PCR (Figure 4.14).

Table 4.5 Putative mutant plants tested for presence of EACMV by PCR after 12- and 32-days post inoculation with EACMV.

Variety	Days post-inoculation (dpi)	Concentration of EMS (%)	Plants with no EACMV detected (% negative PCR plants)
Tajirika	12	0.08%	100
		0.09%	100
	32	0.08%	100
		0.09%	100
Karembo	12	0.08%	100
		0.09%	100
	32	0.08%	57.14
		0.09%	100
Ex-Ndolo	12	0.08%	100
		0.09%	100
	32	0.08%	14.23
		0.09%	100
Mucericeri	12	0.08%	100
		0.09%	100
	32	0.08%	0
		0.09%	100

Seven putative mutant plants were randomly selected and used for detection of EACMV by PCR.

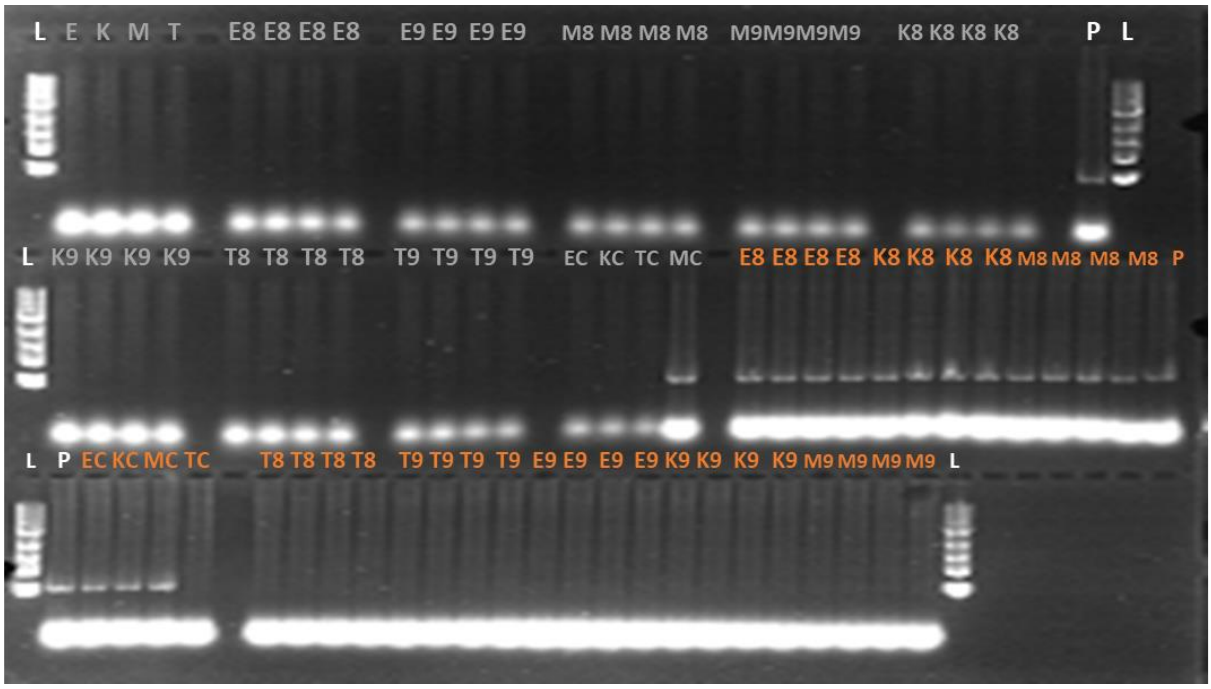


Figure 4.14 Agarose gel electrophoresis image showing PCR amplification of EACMV in putative mutant plants inoculated with EACMV and mock-inoculated control plants.

E, M, K &T represents mock-inoculated plants of Ex-Ndolo, Mucericeri, Karembo and Tajirika confirming that they were virus-free. E8, M8, K8 &T8 and E9, M9, K9 &T9 represents putative mutant plants of Ex-Ndolo, Mucericeri, Karembo and Tajirika derived from stem cuttings treated with 0.08% EMS and 0.09% EMS, respectively. EC, MC, KC &TC represent control plants of Ex-Ndolo, Mucericeri, Karembo and Tajirika that were not treated with EMS but were inoculated with EACMV. L represents 1kb DNA ladder (Bioline, Englands), P represents positive controls. The grey highlighted varieties represent virus indexing at 12 dpi while the red highlighted varieties represent virus indexing at 32 dpi.

4.4 Discussion

Chemical induced mutagenesis is a simple way of inducing genetic variations in plant genomes (Subramaniam and Kumar, 2023). It is one of the effective strategies used for introducing and improving desired traits especially disease resistance in crop plants (Oladosu *et al*., 2016). Among the chemical mutagens, EMS is the most preferred and effective mutagen for inducing mutation in plants due to its high rate of induced mutations and it can form adducts with nucleotides efficiently. EMS predominantly induces single base point mutation (Espina *et al*., 2018). The EMS functions as an alkylating agent by transferring an alkyl group to guanine bases, leading to the production of 6-ethyl guanine. This alteration causes 6-ethyl guanine to pair with thymine instead of cytosine during DNA replication, resulting in G/C to A/T transitions (Sikora *et al*., 2011). These transitions have been observed in over 99% of instances in *Arabidopsis* (Greene *et al*., 2003), 79.8% in maize (Lu *et al*., 2018), and 70% in rice (Till *et al*., 2007). In the present study, chemical induced mutagenesis using EMS was conducted to improve cassava resistance to CMD caused by EACMV.

The sprouting of stem cuttings is one of the most important parameters in determining the effect of mutagens in vegetatively propagated crop plants. In the present study, there was a significant decrease in the duration to sprouting and the sprouting rate in EMS treated stem cuttings of all cassava varieties compared to the non-treated controls. The suppression of sprouting in EMS treated stem cuttings of the tested cassava varieties may be attributed to the metabolic disruption and/or damage of the cell constituents (Ananthaswamy *et al.*, 1971). These results coincide with reports of decrease in survival rate with the increase in EMS concentration in EMS treated stem cuttings in cassava cuttings (Kangarasu *et al*., 2014). The study of rice seeds treated with EMS showed a decrease in survival and stem length with an increase in EMS concentration (Talebi *et*

al., 2012a and 2012b). A remarkable increase in the quantitative characters, including the number of shoots and stem length were detected in putative mutant plants derived from the EMS treated stem cuttings of all test cassava varieties as compared to the control plants from non-treated stem cuttings. The growth stimulatory effect of EMS could be due to a change in hormone signaling response in plant cells (Minisi *et al*., 2013). However, these results contradict the findings from Kangarasu *et al.* (2014) who found a decrease in shoot length, leaf length and width upon treatment with EMS.

The EMS treatment led to changes in leaf characteristics, resulting in mutants with broader leaves. Significant variations in size were noted in potential mutants of Tajirika plant variety following treatment with 0.09% EMS. The leaf mutations may be attributed to damage at the cellular level induced by the mutagen, altered mineral metabolism, and disruptions in the synthesis and transportation of auxins, as indicated by Anjana *et al*. (2012). Consistent with this study, reports of leaf mutants have been documented in various crop plants, including cowpea and faba bean (Khursheed *et al*., 2019; Anjana *et al*., 2002). Mutants displaying broader leaves potentially hold an edge over the parent variety due to their increased leaf area index, which allows them to capture more sunlight for photosynthesis (Konrad *et al*., 2021; Okajima *et al*., 2021). Also, large leaves have fewer partitioning thus the cost of supporting the twigs is lower compared to a plant with higher number of smaller leaves (Wright *et al*., 2017). Large leaves may also be beneficial in cooler climatic zones in the morning hours by stimulating the rate of carbon dioxide assimilation (Okajima *et al*., 2012) and in warm and hot climatic regions during the afternoon by enhancing leaf cooling to avoid leaf damage (Roth-Nebelsick and Krause, 2023).

The findings from this study also revealed that the treatment of stem cuttings with EMS mutagen stimulate the diversity in other morphological traits such as glossy leaves exhibited by putative mutants derived from 0.09% EMS-treated stakes of variety Karembo. This trait is often associated with cuticular wax accumulation (long-chain hydrocarbon compounds) on the plant surface. The change to gloss leaves in the putative mutant plants of Karembo suggests a possible mutation in the genes associated with the cuticular wax biosynthesis pathway. Cuticular wax plays a crucial role as a defense barrier against environmental stress such as drought (Subramaniam and Kumar, 2023). It has been documented that drought stress leads to alterations in the composition and an increase in the quantity of cuticular waxes in *Arabidopsis*, rice, and wheat. Several genes that regulate the biosynthesis and transportation of cuticular wax have the potential to significantly enhance drought tolerance (Li *et al*., 2019). The cuticular wax displayed by the putative mutants of variety Karembo could be an adaptive strategy to enable the plant to be more drought tolerant.

The CMD severity score and disease incidence at 32 dpi was significantly reduced in putative mutant plants stem cuttings of susceptible varieties Ex-Ndolo and Mucericeri treated with 0.09% EMS. At 32 dpi, a total of 8 putative mutants for varieties Ex-Ndolo and 14 of variety Mucericeri were identified as resistant because they exhibited no CMD symptoms and EACMV was not detected in plants tested by PCR. Similar reports by Baguma *et al*. (2021) indicated reduced incidence and severity to CMD in cassava accession BN treated with 0.1 - 0.15% EMS. This reduced level of infection could be due to acquired resistance due to EMS treatment which could have induced cellular fortification or release of phytoalexins and antioxidant enzymes (Baguma *et al*., 2021). The resistance responses observed on this study has also been reported in mutants from different crop plants against viruses for example in *S. lycoperscum* mutants resistant to tobacco mosaic virus (TMV), rice mutants resistant to rice dwarf virus, *A. thaliana* mutants resistant to

TMV, cucumber mosaic virus and turnip mosaic virus (Yamanaka *et al*., 2002; Ishikawa *et al*., 1993; Yoshii *et al*., 2009; Yoshii *et al*., 2004).

4.5 Conclusion

(i) Treatment of cassava stem cuttings with EMS induced morphological mutations that may be of significance in cassava breeding.

(ii) Putative mutant plants derived from cassava stem cuttings treated with 0.09% resulted in significantly reduction in CMD incidence and CMD symptom severity in susceptible varieties at 32 dpi following graft-inoculation with EACMV.

4.6 Recommendations

(i) The level of CMD resistance in putative mutant plants need to be evaluated for a longer period to determine the durability of the resistance.

(ii) The putative mutants need to be analyzed and the genetic mechanisms underlying the morphological changes should be determined in order to genetically improve cassava varieties.

(iii) There is need to carry out next generation sequencing of mutant plants derived from EMS treated cassava stem cuttings to validate the genetic divergence of the mutants from the control plants.

(iv) The genetic stability of the induced mutations should be evaluated after several vegetative generations to eliminate chimeras.

CHAPTER FIVE

5.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General discussion

Cassava is a crucial crop for food security, gaining popularity due to its resilience to challenging climatic conditions that most other cultivated food crops, like cereals lack. It is the fourth most significant staple food crop, following rice, wheat, and maize (Rey and Vanderschuren, 2017; Tafesse *et al*., 2021). More than 800 million people worldwide, with half of them living in Africa, rely on cassava as a source of nutritional starch (Alabi *et al*., 2011). However, there are numerous obstacles to cassava production, the primary one being CMD which poses a threat to cassava in all growing regions. Several cassava geminiviruses cause CMD which can reduce yield in 95% of farmed plants in susceptible varieties (Fauquet and Fargette, 1990).

To mitigate the disease in farmers' crops, the best approach is to utilize resistant cassava cultivars and provide clean planting materials. It has been revealed that cassava cultivars enhanced for CMD resistance contain three CMD resistance genes: CMD1, CMD2, and CMD3. But according to Ndunguru *et al*. (2016), there have been instances when the resistance has broken down, and according to Amoakon *et al*. (2023), certain resistant varieties do not carry the three resistance genes. There is therefore a need to explore additional sources of genetic resistance to CMD in cassava as well as methods of eliminating geminiviruses from susceptible but farmer preferred varieties. In the present study, natural resistance (R) genes, phytosanitation and mutation were explored for their effectiveness in eliminating and inducing resistance to CMGs.

The use of plant host genes with antiviral activity is one of the potential strategies to build genetic resistance to viruses. Previous studies indicate several functional NBS-LRR genes involved in disease resistance in many plant species (Neupane *et al*., 2018; Goyal *et al*., 2020; Zhang *et al*., 2020; Shi *et al*., 2018; Liu *et al*., 2021). Therefore, NBS-LRR genes are important sources for mining functional resistance (R) genes for CMD in cassava. Natural resistance (R) genes conferring resistance to viruses in other crop plants were selected, homologs from cassava identified and characterized in the study. The identified genes belonged to the CC-NBS-LRR class of R genes that account for 80% of the R genes in plants. The seven genes in the study were classified into three evolutionary groups and all had different conformation in their tertiary structure. Fifteen different motifs were identified from the genes but six of them were common in all the genes thus showing that they exhibited conserved motifs. The expression patterns of selected NBS-LRR genes were analyzed to determine the transcriptional response following EACMV infection. The genes were differentially expressed in susceptible and resistant cassava varieties with the expression being highest in the resistant varieties. Notably, the two genes namely manes.03G063225 and manes.11G004600 were expressed in resistant varieties throughout the infection period (67 days) suggesting that they could have protective effects against CMD for a longer period of the disease response. The findings demonstrated that the up regulated genes are involved in resistance response and could provide invaluable sources of disease resistance to molecular breeders in future.

Cassava is a vegetatively propagated crop and therefore accumulation of viruses in susceptible varieties occurs over generations limiting the supply of clean and healthy planting materials to farmers. The use of clean planting materials has been reported to mitigate the effects of viruses in

farmers' fields (Dennien, 2015). Chemotherapy using SA and H_2O_2 and thermotherapy using HW was carried out in this study to determine their effectiveness in eliminating CMGs from infected cassava stem cuttings on-field. The results of the study showed that CMD-infected stem cuttings subjected to SA at concentration of 5 mM for 6 h was the most effective in eliminating CMGs with an efficiency of 65.1%, whereas H_2O_2 treatment of 1% for 12 h eliminated CMGs with an efficiency of 77.8%. HW treatment of 50 ºC for 5 min eliminated the virus in 81.7% of the plants derived from treated stem cuttings. Salicylic acid is a phytohormone that induces systemic acquired resistance in plants (Madhusudhan *et al*., 2009) and therefore at the correct dose, it could serve as a potential chemical for inducing viral resistance in plants. Hydrogen peroxide acts in a similar way as SA by inducing system acquired resistance (SAR), creating a hypersensitive response that leads to destruction of the viral particles, prevents viral replication and movement thus preventing entry into the meristem from the neighboring cells (Hernández *et al*., 2016; Liao *et al*., 2013). Therefore, H_2O_2 has the potential to eradicate CMGs from infected cassava planting material. Hot water therapy at the right temperature and exposure time makes the environment unfavorable for viral replication by destruction of the coat protein of viruses. The methods described in this study were performed on-field in pots and therefore can easily be adopted by smallholder farmers since they can be applied directly on the stem cuttings before planting thus providing farmers with clean planting materials. The methods could also play a significant role in the exchange of cassava stem cuttings for international trade.

The analysis of antioxidant enzymes (catalase, peroxidase and ascorbate peroxidase) showed that the activity of enzymes significantly increased in virus eliminated cassava plants than the control virus infected plants demonstrating the role of these enzymes in defense against CMGs. Catalase and ascorbate peroxidase act in the first line of defense by detoxifying excess amounts of H_2O_2

and therefore maintaining a balance of H_2O_2 because plants also utilize this molecule as signal molecule in regulating stress responses. Peroxidase has antiviral property that destroys the virus in host tissues. Increased levels of antioxidant enzymes may probably have a correlation with the elimination of viruses from plants using chemotherapy and thermotherapy and therefore these enzymes could be used as biochemical markers. Hydrogen peroxide and Malondialdehyde (MDA) were more liberated in the infected cassava plants than in the plants where CMGs were eliminated. This indicates the level of stress due to virus infection in plants and their high levels are destructive to plant tissues. High levels of MDA lead to lipid peroxidation, whereas H_2O_2 in high amounts leads to chlorosis of plant leaves.

The use of chemical mutagens has been used in plant breeding to develop new germplasm, increase crop yields, nutritional quality and resistance to pests and diseases (Ishtiaq *et al.*, 2023). The ethyl methyl sulfonate (EMS) is one of the most potent and useful chemical mutagens, which causes random point mutations with minimal loss and/or deletion of chromosomal region in plants. In this study, mutation breeding was exploited for induction of genetic resistance to CMGs by treating cassava stem cuttings with EMS chemical mutagen at a concentration of 0.08% and 0.09%. The results demonstrated decreased sprouting of stem cuttings treated with EMS due to the effects of the mutagen at the physiological and cellular level of the plant tissues. The findings demonstrated that the use of the chemical mutagen at a dose of 0.09% was effective in inducing resistance to CMGs by reducing the disease incidence and reducing disease severity in cassava. Putative mutant plants of variety Tajirika exhibited large leaves compared to the control plants. Putative mutant plants of variety Karembo had glossier leaves probably due the mutation genes involved in cuticle biosynthesis leading to increase in cuticular wax production. Also, putative mutant plants of EMS

treated stem cuttings showed enhanced growth parameters including stem length and number of shoots. Thus, EMS has the potential of inducing morphological mutations in cassava plants.

5.2 Conclusions

(i) The seven resistance (R) genes might be involved in response of cassava to EACMV infection based on their expression profiles in resistant and susceptible cassava varieties.

(ii) The use of salicylic acid (SA), hydrogen peroxide (H_2O_2) and hot water (HW) treatment of cassava cuttings at the optimum dose and duration are effective in eliminating cassava geminiviruses in CMD-infected stem cuttings thus providing an easy and affordable approach in obtaining clean seed for farmers.

(iii) The putative plants derived from EMS treated cassava stem cuttings displayed morphological variation, reduced CMD incidence and severity compared to control plants derived from non-treated stem cuttings.

5.3 Recommendations

(i) There is a need to study the gene function and disease resistance functions of the selected resistance genes as candidates for virus resistance in cassava breeding programs.

(ii) Further studies are required to determine the effect of cassava variety in eradication of geminiviruses from CMD-infected plants in response to SA, H_2O_2 and HW treatments.

(iii) The effect of different treatment combinations such as HW with SA in eradication of geminiviruses from infected plants needs to be further evaluated.

(iv) Extensive screening and characterization of novel mutants generated in this study for future use in cassava breeding for virus resistance is important.

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