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ORIGINAL ARTICLE

Reduction of cassava mosaic geminiviruses from infected stem cuttings using salicylic acid, hydrogen peroxide and hot water treatment

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Abstract

Cassava mosaic disease (CMD) causes significant losses in cassava production in Africa. The disease is caused by several cassava mosaic geminiviruses (CMGs) and spread through the use of infected plant materials. The infected plants remain infected throughout their vegetative lifecycle as the disease cannot be controlled by standard plant protection measures. Therefore, it is important to develop inexpensive field-based methods that can be easily adopted by small-scale farmers to sanitize geminiviruses-infected stem cuttings used as planting material. This study aimed at eliminating CMGs from infected cassava stem cuttings by use of salicylic acid (SA), hydrogen peroxide (H_2O_2) and hot water (HW) treatments. Eight varieties of CMD-infected stem cuttings were pretreated with SA (1.25, 2.5 and 5 mM), H_2O_2 (0.5%, 1.0% and 1.5%) or hot water (50 and 55°C) at different exposure times before establishment in the glasshouse for evaluation of subsequent plant growth and geminivirus detection. Based on the cassava varieties used for the different treatments, treatment with hot water at 50°C for 5 min and 1.0% H_2O_2 for 12 h were the most effective in eliminating CMGs with an efficiency of 81.7% and 77.8%, respectively. Salicylic acid at a concentration of 5 mM for 6 h eliminated the viruses in 65.1% of the cuttings. The methods tested herein have the potential for producing planting materials with significantly reduced CMD risk for smallholder farmers and the cassava industry to meet their increasing demand. It could also contribute to the global exchange of germplasm for conservation and breeding programs.

KEYWORDS
cassava mosaic virus, hot water thermotherapy, hydrogen peroxide, salicylic acid, stem cuttings, virus elimination

1 | INTRODUCTION

Cassava (*Manihot esculenta*) is a food security crop mainly grown by resource-limited farmers in sub-Saharan Africa due to its high starch content and its ability to resist adverse conditions (Alabi et al., 2008). However, the crop is faced by a myriad of biotic constraints that affect its yield and quality. Among the major biotic constraints affecting the crop is cassava mosaic disease (CMD) (Mohammed

et al., 2012; Thresh & Cooter, 2005). The disease is caused by several cassava mosaic geminiviruses (CMGs) (genus *Begomovirus*, family *Geminiviridae*) of which African cassava mosaic virus (ACMV), East African cassava mosaic virus (EACMV) and South African cassava mosaic virus (SACMV) are among the species that cause major losses on the African continent (Jacobson et al., 2018; Zerbini et al., 2017). CMD leaf symptoms in infected cassava are characterized by green to yellow mosaic, often with leaf margin distortion. 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 t which is equivalent to a $1.2–2.3 billion USD loss annually (FAOSTAT, 2020). In susceptible cultivars, CMD can cause estimated yield losses of 20%–95% (Arama et al., 2016; Legg et al., 2015; Tomlinson et al., 2019). The CMD-associated geminiviruses cannot be controlled by conventional plant protection measures, and thus planting healthy vegetative propagation material is crucial for sustainable cassava production.

CMGs are propagated by infected planting material and transmitted/spread by the whitefly vector, *Bemisia tabaci* (Gennadius) (Duraisamy et al., 2013; Macfadyen et al., 2018; Maruthi et al., 2005). The vegetative nature of cassava stem cuttings makes them prone to the dissemination of CMD. Methods to clean CMG infections, such as thermotherapy and somatic embryogenesis (Maruthi et al., 2019; Mutai et al., 2017; Nakabonge et al., 2020; Zinga et al., 2023) have so far been developed 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 & Nakabonge, 2016). Therefore, field-based methods to sanitize geminivirus-infected stem cuttings need to be developed to meet the increasing demand of disease-free planting material.

High temperatures are known to inhibit replication of several plant viruses, and the use of high-temperature treatments has been reported to be effective in elimination of viruses in vegetatively propagated plants through in vitro culture of plantlets (Chellappan et al., 2005; Obrępalska-Stęplowska et al., 2015). The high temperatures in heat therapy hinder viral replication, thus compromising their survival (Nangonzi et al., 2016). Hot water (HW) treatment as a method of eliminating viruses in plants requires less skill and non-sophisticated laboratory facilities, making it cost-effective. Furthermore, HW treatment has been reported to be effective in eliminating viruses from plants (Abbas et al., 2016; Ling, 2010; Nangonzi et al., 2016).

Phytohormones are induced in plants upon pathogen attack to enhance resistance (Collum & Culver, 2016; Ghosh & Chakraborty, 2021; Gupta et al., 2022; Zhao & Li, 2021; Zhong et al., 2021). Researchers have used this knowledge by applying phytohormones exogenously to plants to enhance their resistance to pathogens. Salicylic acid (SA) is one such phytohormone whose accumulation is enhanced in several resistant plant–virus interactions causing both systemic and localized hypersensitive response (HR) resistances (Baebler et al., 2014; Jovel et al., 2011). The SA pathway is triggered by plants upon pathogen attack, initiates HR and inhibits viral replication, viral movement as well as coat protein accumulation (Murphy et al., 2020). Some of the defence responses implicate production of antimicrobial compounds and accumulation of reactive oxygen species (ROS). H_2O_2 is a ROS that plays a double role by stimulating localized cell death to inhibit the pathogen, and by production of signals that trigger antioxidant and pathogenesis-related (PR) genes in neighbouring plant tissues. Some PR proteins function as signal molecules in the initiation of systemic acquired resistance (SAR). H_2O_2 induces gene expression and production of enzymes needed in defence of plants against viruses and is a ROS released upon infection of plants by pathogens (Mejía-Teniente et al., 2019; Radwan & Ismail, 2020).

The aim of the present study was to evaluate the CMG-reducing effects of exogenous treatment of cassava stem cuttings, used as planting material, with SA, H_2O_2 and HW.

2 | MATERIALS AND METHODS

2.1 | Plant material

Stem cuttings of 9-month-old plants of eight cassava varieties exhibiting CMD symptoms were collected from fields at the 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 the Houngue et al. (2019) severity score scale where 1 = plants with no symptoms on the leaves; 2 = plants with mild chlorotic spots or some distortion at the base of leaves; 3 = plants with severe chlorotic spots on the entire leaf surface with leaf twisting; 4 = plants with distorted or shrunken leaf blades (2/3 of the leaf area); and 5 = plants with severe symptoms of CMD and/or total distortion of 4/5 of the leaf area and stunting of the whole plant.

2.2 | Confirmation of cassava mosaic geminiviruses in plants of the selected cassava varieties

The presence of cassava mosaic geminiviruses (ACMV and EACMV) in plants of the selected varieties was confirmed by polymerase chain reaction (PCR) using primers specific for ACMV (CMB-Rep F – CRTCAATGACGTTGTACCA and ACMV-Rep R – CAGCGGMAGTAAGTCMGA) and EACMV (CMB-Rep F – CRTCAATGACGTTGTACCA and EACMV-Rep R – GGTTTGCAGAGAACTACATC) as described by Alabi et al. (2008). Total genomic DNA was extracted using the cetyltrimethylammonium

bromide (CTAB) method as described by Osena et al. (2017). The DNA pellet was re-suspended in nuclease-free PCR-grade water, and the concentration of DNA in each sample was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and adjusted to 50 ng/μL. The extracted DNA was then subjected to PCR using specific primers for the detection of ACMV and EACMV. The PCR reaction was prepared in a final reaction volume of 25 μL consisting of 12.5 μL Quick-Load Taq 2× master mix (New England Biolabs), 0.5 μL of 10 μM of each of the reverse and forward primers, 2 μL of DNA template and 9.5 μL of nuclease-free PCR-grade water. The PCR was carried out in conventional thermocycler (Eppendorf AG 22331, Hamburg, Germany). The PCR temperature profile was as follows: 95°C for 3 min for initial denaturation followed by 30 cycles of amplification at 95°C for 30 s, 55°C for 30 s, 72°C for 60 s for both primers and final elongation step at 72°C for 10 min. PCR-amplified products were separated by 1% agarose gel electrophoresis and stained with 0.2× GelRed nucleic acid stain (Biotium, Fremont, CA). Gel images were visualized under UV using gel documentation system (Azure C200, Azure Biosystems, Dublin, CA). The stem cuttings of ACMV- and EACMV-positive plants were used for SA, H_2O_2 and HW treatment experiments.

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

Stem cuttings of varieties MM96/5280, Fumbachai and Nanzala were used for SA treatments. Cultivars Matuja, Nyadai and Serere were used for H_2O_2 treatment, whereas cultivars Magana, Abarobaka and Nyadai were used for HW treatment. Since different cultivars were used for different sanitation methods, cultivar x treatment interactions may prevent direct statistical comparisons of treatment efficacies. Cuttings were subjected to different concentrations of SA (1.25, 2.5 and 5 mM) for 6, 12 and 24 h; different concentrations of H_2O_2 (0.5%, 1.0% and 1.5%) for 6, 12 and 24 h; and different hot water temperatures (50 and 55°C) for 5 and 10 min. Nine stem cuttings (approximately 4–5 nodes long) per replicate were subjected to each treatment combination (concentration/temperature × exposure time), and each treatment combination was replicated three times; thus, a total of 27 stem cuttings were used for each treatment combination. Sterile distilled water was used as a control. All treatment combinations and non-treated control stem cuttings were planted in 2 litre plastic pots in a completely randomized design.

2.4 | Establishment of treated stem cuttings and phenotypic analysis of established plants in the glasshouse

2.4.1 | Experimental procedure

A total of 27 stem cuttings for each variety were used for each treatment combination. The experimental design comprised three biological replicates comprising nine plants each per treatment combination. The nine plants were divided into three separate groups of three pooled plants (technical replicates).

Data on stem length, number of shoots, virus elimination efficiency, virus score and disease incidence were collected from the 27 plants from each experiment. For the biochemical assay, from the three technical replicates, three leaves were collected and pooled together for analysis to form a replicate thus adding up to three replicates.

2.4.2 | Treatment protocols

The stem cuttings treated with SA, H_2O_2 and HW were planted in 2 litre plastic pots (Kenpoly, Kenya) containing sterilized loamy soil mixed with sterilized farmyard manure in the ratio of 1:3 (v/v) and placed in controlled conditions (28°C and 70% relative humidity) in a glasshouse. Survival was evaluated 2 weeks after treatments by counting the number stem cuttings that sprouted shoots. 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 of sprouted leaves was scored using a severity scale of 1–5 according to Houngue et al. (2019). The plants were scored for CMD severity at 2, 4, 6 and 8 weeks after sprouting.

2.5 | Detection of ACMV and EACMV in sprouted plants

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 topmost leaves that exhibited CMD symptoms were sampled for genomic DNA extraction and subsequent PCR analysis as described earlier. Results were analysed 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).

2.6 | Analysis of hydrogen peroxide and lipid peroxidation in treated plants

Newly emerged leaves of plants without CMD infection after treatment of stem cuttings with SA, H_2O_2 and HW and untreated control plants with CMD infection were analysed for the levels of H_2O_2 and malondialdehyde (MDA) after 8 weeks in the glasshouse. The amount of H_2O_2 was determined by measuring the optical density using potassium iodide as delineated by Velikova et al. (2000). Leaves making up 0.5 g were homogenized with a mortar and pestle in 2 mL of ice-cold 0.1% (w/v) trichloroacetic acid (TCA). The suspension was centrifuged at 10,000 rpm for 30 min at 4°C. To 0.5 mL of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide were added. The absorbance was

read at 390 nm and the amount of H_2O_2 was calculated using the extinction coefficient 0.28 $mM^{-1} cm^{-1}$ and expressed as μmol/g fresh weight (FW).

Lipid peroxidation was estimated by the amount of MDA produced using the method described by Hodges et al. (1999) and Chen and Gallie (2006) based on thiobarbituric acid (TBA). In 2 mL of 0.1% (w/v) TCA solution on ice, 0.1 g leaf sample was homogenized. The suspension was centrifuged at 10,000 rpm for 5 min, and 0.5 mL of the supernatant was collected. To the supernatant, 1 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA was added and the mixture was incubated in a water bath at 95°C for 30 min and quickly cooled on ice. The mixture was centrifuged at 10,000 rpm for 10 min and the absorbance was measured at 532 and 600 nm. Non-specific turbidity was corrected by subtracting the absorbance at 600 nm from that at 532 nm. The concentration of MDA was calculated with its extinction coefficient 155 $mM^{-1} cm^{-1}$ and expressed as μmol/g fresh weight.

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 in untreated control plants with CMD infection. The number of biological and technical replicates was as for the H_2O_2 accumulation assay described above. Leaf samples weighing 0.5 g were homogenized using a pestle and mortar in 2 mL of buffer (1% w/v polyvinylpyrrolydone (PVP), 0.2 mM EDTA and 100 mM potassium phosphate buffer pH 6.8) on ice. The mixture was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatants were assayed for catalase, peroxidase and ascorbate peroxidase (APX) enzyme activities.

Ascorbate peroxidase activity was assayed by measuring the oxidation of ascorbic acid and recording the change at an absorbance of 290 nm. The leaf extract of 10 μL was mixed with 1 mL of a reaction buffer containing 0.25 mM ascorbic acid, 0.2 mM Tris/HCl buffer (pH 7.8) and 0.5 mM H_2O_2. APX activity was calculated from the extinction coefficient (2.8 $mM^{-1} cm^{-1}$) of ascorbate (Nakano & Asada, 1981).

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

Peroxidase (POD) activity was determined by adding 50 μL of leaf homogenate to 2 mL of the reaction mixture containing 25 mM H_2O_2, 25 mM guaiacol and 50 mM sodium acetate buffer (pH 7). The activity of POD was determined by recording the absorbance at 470 nm which increases due to the formation of tetra guaiacol (coefficient of extinction 26.6 $mM^{-1} cm^{-1}$). The peroxidase activity was expressed in Units/g FW.

2.8 | Data analyses

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 (treatment concentration/temperature × treatment time × variety arranged in factorial design), whereas data on oxidative stress markers, antioxidant enzyme activities, survival percentage, disease incidence and virus elimination efficiency were subjected to one-way ANOVA arranged in factorial design using GenStat software version 15. Comparisons of the means were done using Tukey's HSD test at 5% probability level. Graphs and tables with the means were generated using the Genstat software.

3 | RESULTS

3.1 | Detection of cassava geminiviruses in plants of the selected cassava varieties

The virus status of the eight cassava cultivars was screened using PCR and the PCR products visualized by agarose gel electrophoresis (Figure 1). The expected amplicon size was 368 bp for ACMV and 650 bp for EACMV. Of the leaf samples tested, cultivars Matuja, Magana, MM96/5280, Nanzala and Abarobaka were co-infected with ACMV and EACMV, cultivar Serere was singly infected with EACMV, whereas cultivars Nyadai and Fumbachai were singly infected with ACMV (Figure 1). The stem cuttings of ACMV- and EACMV-positive plants were used for SA, H_2O_2 and HW treatments for virus elimination.

3.2 | Effect of treatments on survival and growth performance of established plants

The survival 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 those treated with SA at 5 mM for 24 h had the least survival ranging from 22.0% to 44.4% (Table 1). As the concentration and exposure time to SA increased, the survival percentage significantly decreased for all the tested cassava varieties (Table 1). 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 with the untreated controls ($p \leq .05$) (Table 2).

The survival percentage of plants from stem cuttings treated with 0.5% H_2O_2 for 6 and 12 h was the highest, ranging from 87.7% to 100% (Table 1). 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 tested cassava varieties. As the concentration and exposure period of H_2O_2 increased, the survival percentage significantly decreased (Table 2). The length of the stem was reduced in plants from stems treated with H_2O_2 compared with

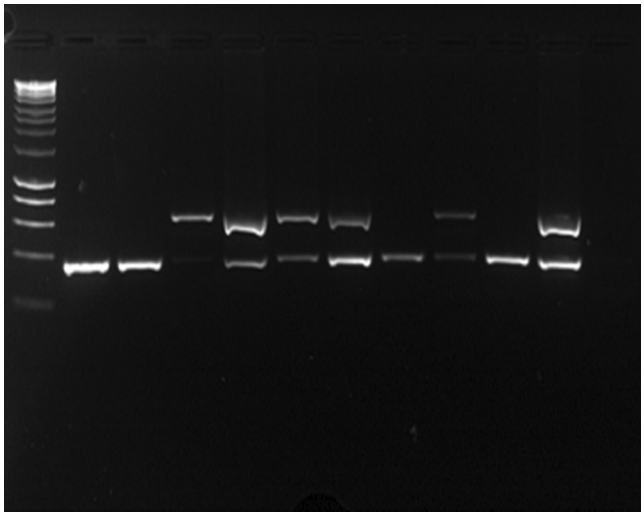

FIGURE 1 Agarose gel electrophoresis showing PCR amplification of DNA from leaves of cassava mosaic virus disease (CMD) infected plants of 8 cassava cultivars used for phytosanitation and representative CMD-infected cassava plant showing stem cuttings used for sanitation experiment. Detection of African cassava mosaic virus and East African cassava mosaic virus in infected cassava plants using primer set CMB-Rep F & ACMV-Rep R which amplifies ACMV (368 bp) and CMB-Rep F & EACMV-Rep R which amplifies EACMV (650 bp). L represents 1 kb DNA Hyperladder (Bioline, London, England); 1–6 represents samples from plants of cultivars 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.

their controls across all the varieties. The number of shoots was significantly reduced ($p \leq .05$) in plants sprouted from stem cuttings treated with H_2O_2 compared with the untreated controls (Table 2).

The highest survival percentage of between 77.8% and 100% was recorded in sprouted plants from stem cuttings of the three cassava varieties treated with hot water at 50°C 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°C for 10 min for all the three varieties (Table 1). As the water temperature increased, the survival of sprouted plants was significantly compromised ($p \leq .05$). Overall, sprouted plants from stem cuttings treated with HW at 55°C showed a highly significant reduction in the length of the stems compared with those treated with HW at 50°C (Table 2). Plants from stem cuttings treated with HW at 55°C showed the highest decrease in the number of shoots compared with those treated with HW at 50°C (Table 2).

3.3 | Symptomatology of sprouted plants from treated stem cuttings

Characteristic CMD symptoms including chlorosis, leaf distortion and leaf curling/misshapen leaf blades were observed in all untreated control plants of all the cassava varieties (Figure 2). The onset of minor CMD symptoms in sprouted plants from stem cuttings treated with H_2O_2, SA and HW were delayed compared with the untreated controls.

3.4 | CMD severity in sprouted plants from treated stem cuttings

Sprouted plants from 5 mM SA-treated stem cuttings for 6 h showed a significant reduction in symptom severity score compared with the untreated controls for all the cultivars (Figure 3a). The disease symptom severity score was lowest in sprouted plants from stem cuttings treated with 1% H_2O_2 for 12 h (Figure 3b). The CMD symptom severity score was significantly reduced in all plants of stem cuttings treated with hot water compared with the untreated controls (Figure 3c).

3.5 | CMD incidence in sprouted plants from treated stem cuttings

Sprouted plants from stem cuttings treated with 1% H_2O_2 for 24 h had the highest disease incidence of CMD at 86.7% followed by 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). The disease incidence was highest (100%) in plants from stem cuttings treated with 2.5 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). Plants from stem cuttings treated with HW at 55°C 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°C for 5 min (Table 3).

3.6 | Efficiency of virus elimination in plants from treated stem cuttings and PCR verification of CMGs

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 4). For SA, a concentration of 5 mM for 6 h was the most effective in eliminating CMGs at an efficiency of 65.1% (Table 4). For HW, treatment at 50°C for 5 min was the most effective in eliminating CMGs with an efficiency of 81.7% (Table 4). The percentage of virus-infected plants after all the treatments ranged from 0% to 100% (Table 4). However, none of the treatments could completely eradicate CMGs from infected

TABLE 1 Survival percentages of sprouted plants from cassava stem cuttings treated with hydrogen peroxide (H_2O_2), salicylic acid (SA) and hot water (HW) at different concentrations/temperature and exposure times.

Variety	Time (h)	H_2O_2 conc. (%)	0	0.5	1.0	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			100 ± 0			
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
Control			100 ± 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 ± 0			
Variety	**Time (h)**	**SA conc. (mM)**	**0**	**1.25**	**2.5**	**5.0**
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
Control			100 ± 0			
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
Control			100 ± 0			
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	
Control			100 ± 0			
Magana	5			81.5 ± 9.8	55.6 ± 12.8	
	10			88.9 ± 11.1	55.6 ± 11.1	
Control			100 ± 0			
Nyadai	5			77.8 ± 0	55.6 ± 6.42	
	10			66.7 ± 6.42	55.6 ± 11.1	
Control	0		100 ± 0			

Note: Values are means and standard errors. The experiments were initiated using nine stem cuttings for each treatment combination (concentration/temperature × exposure time), and the experiments were replicated three times.

stem cuttings. Elimination of CMD using HW was the most effective treatment.

The efficiency of elimination of CMGs from plants obtained from SA, H_2O_2 and HW-treated stem cuttings grown in the greenhouse for 8 weeks was analysed using PCR. After treatment with SA, H_2O_2 and HW, all sprouted plants that had no visible symptoms in the glasshouse tested negative for CMD based on PCR amplification, whereas those with symptoms in the glasshouse 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.

3.7 | Analysis of hydrogen peroxide and lipid peroxidation in treated plants

The amount of H_2O_2 and MDA was significantly higher ($p \leq .05$) in sprouted plants from the untreated stem cuttings (controls)

TABLE 2 Growth parameters (stem length and number of shoots) of sprouted plants from cassava stem cuttings treated with hydrogen peroxide (H_2O_2), salicylic acid (SA) and hot water (HW) at different concentrations/temperature and exposure times.

Variety	Time (h)	H_2O_2 conc. (%)	Stem length (cm)				No. of shoots			
			0	0.5	1.0	1.5	0	0.5	1.0	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**	**0**	**1.25**	**2.5**	**5.0**
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			

Note: Values are means and standard errors. The experiments were initiated using nine stem cuttings for each treatment combination (concentration/temperature × exposure time) and the experiments were replicated three times.

compared with stem cuttings that CMGs were successfully eliminated with HW, SA and H_2O_2 treatments (Figure 4). 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 untreated control stem cuttings (Figure 4).

3.8 | Changes in the activities of catalase, peroxidase and ascorbate peroxidase enzymes

The activity of the antioxidant enzymes CAT, POD and APX was significantly higher ($p \leq .05$) in the plants where CMGs were successfully eliminated with HW, SA and H_2O_2 than in the untreated

FIGURE 2 Cassava plants established in plastic pots after salicylic acid (SA) treatment, hydrogen peroxide (H_2O_2) treatment, Hot water (HW) treatment and symptomatic CMD-infected untreated control plants (plants treated with distilled water). (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 untreated control plants. (c) Asymptomatic cassava plants after treatment with 1% H_2O_2 for 12 h; (d) characteristic CMD symptoms (severe/mild leaf chlorosis, leaf distortion and twisting), on untreated control plants. (e) Asymptomatic cassava plants after treatment with HW at 50°C for 5 min; (f) characteristic CMD symptoms (severe/mild leaf chlorosis, leaf distortion and twisting) on untreated control plants (plants treated with distilled water at room temperature).

stem cuttings (Figure 5). There was no significant difference in the activities 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 untreated control stem cuttings (Figure 5).

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 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 among the most efficient strategies to control plant diseases through induction of enhanced plant resistance known as SAR which is associated with the expression of plant defence genes. SA is one such chemical inducer and plays a key role in plant defence mechanisms against pathogens (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 from stem cuttings treated with 5 mM SA for 6 h were lower than that from stem cuttings treated with 1.25 and 2.5 mM for 6, 12 and 24 h and that treated with 5 mM SA for 12 and 24 h. This result is similar to previous reports by Ong and Cruz (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. Furthermore, Li et al. (2019) also reported that exogenous application of SA in tomato induced resistance to the geminivirus, tomato yellow leaf curl virus. In this study, growth of sprouted plants from cassava

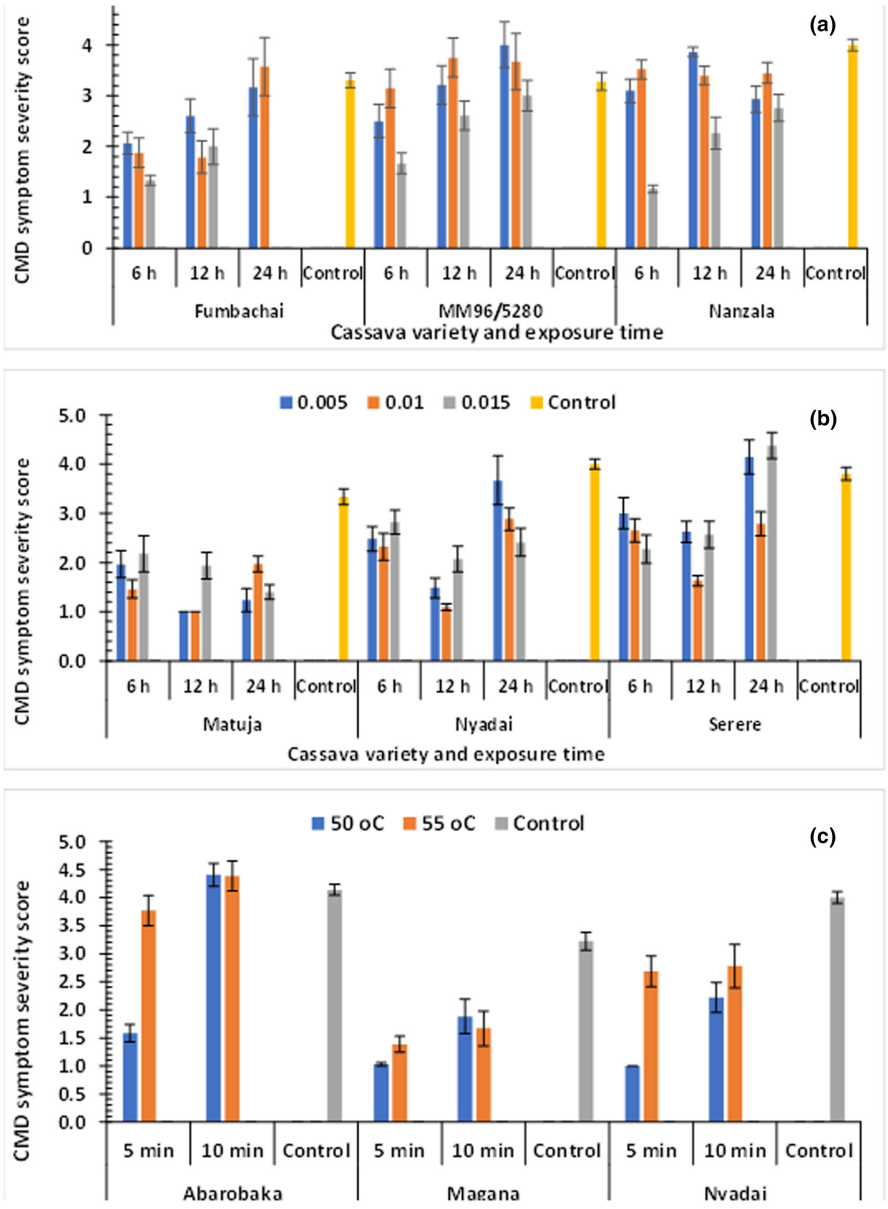

FIGURE 3 CMD severity scores of sprouted plants of different cassava cultivars obtained from stem cuttings treated with salicylic acid (SA) (a), hydrogen peroxide (H_2O_2) (b) and hot water (HW) (c) at different exposure times. The values are means of three replicates ± standard deviation (SD).

stem cuttings treated with SA was significantly reduced compared with that of the untreated 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 contradict the findings of Zhang and Li (2019) and Xi et al. (2021), whereby SA promoted plant growth rather than suppressing the growth as was the case in this study. This demonstrates that the effect of SA on plant growth and virus levels may be host–virus interaction specific.

Hydrogen peroxide is also one of the chemical inducers of SAR, which in turn signals the activation of defence genes and the formation of phytoalexins (Hernández et al., 2016; Kuźniak & Urbanek, 2000; Liao et al., 2013; Orozco-Cardenas & Ryan, 1999). It has been reported that H_2O_2 plays a signalling role in plants under stress and pathogen defence, particularly against virus infection (Radwan & 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 and growth of plants. These data suggest that H_2O_2 affects cassava survival depending on the dose of H_2O_2 used. H_2O_2 can elicit localized cell death in plants and leads to lipid peroxidation that damages the cell membrane; hence, the reduction in survival 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

TABLE 3 Cassava mosaic disease incidence and symptomatology of sprouted plants from cassava stem cuttings treated with hydrogen peroxide (H_2O_2), salicylic acid (SA) and hot water (HW) at different concentrations/temperature and exposure times.

Time (h)	H_2O_2 conc. (%)	0	0.5	1.0	1.5	Symptoms of sprouted plants
6			56.0de	56.6de	79.6bc	Severe/mild chlorosis, leaf twisting and distorted leaf blades
12			42.59e	22.2f	66.7cd	Mild/severe chlorosis, leaf distortion and leaf twisting
24			83.33b	86.7ab	83.3b	Severe chlorosis, leaf twisting and distorted leaf blades
Control		100a				Severe chlorosis, leaf twisting and distorted leaf blades
Time (h)	**SA conc. (mM)**	**0**	**1.25**	**2.5**	**5.0**	
6			82.3bc	79.4c	34.9d	Severe/mild chlorosis, leaf twisting and distorted leaf blades
12			95.24ab	81.4bc	84.4bc	Severe/mild chlorosis, leaf twisting and distorted leaf blades
24			93.3abc	100a	100a	Severe chlorosis, leaf twisting and distorted leaf blades
Control		100a				
Time (min)	**HW Temp. (°C)**	**0**	**50**	**55**		
5			43.2b	73.2b		Mild/severe chlorosis and leaf twisting
10			47.7c	77.7a		Severe chlorosis and leaf twisting
Control		100a				Severe chlorosis, leaf twisting and distorted leaf blades

Note: Values are means and standard errors. Means followed by the same letters within a given treatment are not significantly different ($p = .05$). The experiments were initiated using nine stem cuttings for each treatment combination (concentration/temperature × exposure time) and the experiments were replicated three times.

exposure time and concentration 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.

Based on the cassava variety used, HW treatment of cassava stem cuttings at 50°C 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°C 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 (Nangonzi et al., 2016). While the survival of the cassava plants at 50°C for 5 min was highest at 77.8% to 100% followed by that of 50°C for 10 min at 66.7% to 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°C also showed a decrease in disease incidence to 25% in CMD-infected plants (Nangonzi 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 crowns after HW treatment at 58°C for 40 min. Higher temperatures damage 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 (Damayanti & Putra, 2010; Ling, 2010), and in these cases this type of treatment may not be applicable to eliminating viruses.

The levels of H_2O_2 and lipid peroxidation were higher in CMG-infected cassava compared with the virus-eliminated cassava plants. H_2O_2 is released in plant cells in high amounts upon abiotic or biotic stress, acting as a signalling molecule to induce pathogen-related defence 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 the formation of radical intermediates (Riedle-Bauer, 2000).

TABLE 4 Virus elimination efficiencies from sprouted plants after treatments of cassava stem cuttings with hydrogen peroxide, (H_2O_2), salicylic acid (SA) and hot water (HW).

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

Note: Values are means and standard errors. Means followed by the same letters within a given treatment are not significantly different ($p = .05$). The experiments were initiated using nine stem cuttings for each treatment combination (concentration/temperature × exposure time), and the experiments were replicated three times.

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 concentrations of H_2O_2 (Terzi et al., 2018). CAT works in the first line of defence by breaking down H_2O_2 to water and oxygen and has low affinity for H_2O_2 and 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, 2006; Baker & Orlandi, 1995). POD is the first enzyme that shows quick defence 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). In this study, the levels of H_2O_2 in the CMD-infected controls remained high due to downregulation 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 with uninfected tomato and bell pepper (Li et al., 2019; Ong & Cruz, 2016; Xi et al., 2021). These plants also showed an accumulation of H_2O_2 and higher levels of lipid peroxidation.

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 since there were not enough stem cuttings of the same variety infected with the target CMGs in the field to carry out sufficient experimental replicates. Thus, the different treatments applied to different cassava varieties cannot be compared directly, although statistical comparisons of application concentrations, temperatures and/or times within a given treatment are feasible. Further studies are required to determine the effect of cassava variety in eradication of geminiviruses from infected plants

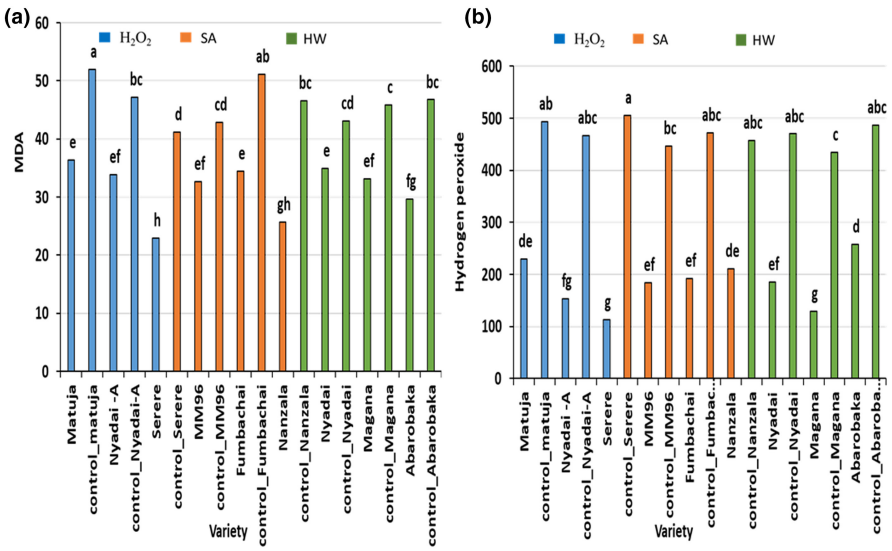

FIGURE 4 The levels of (a) malondialdehyde (MDA) (μmol MDA g^{-1}FW) and (b) hydrogen peroxide (H_2O_2) (μmol H_2O_2 g^{-1}FW) in the virus-eliminated plants from SA, H_2O_2 and HW-treated stem cuttings and plants from the untreated control stem cuttings. The values are means of three biological replicates repeated three times (three technical replicates). Similar letters in the error bar show no significance.

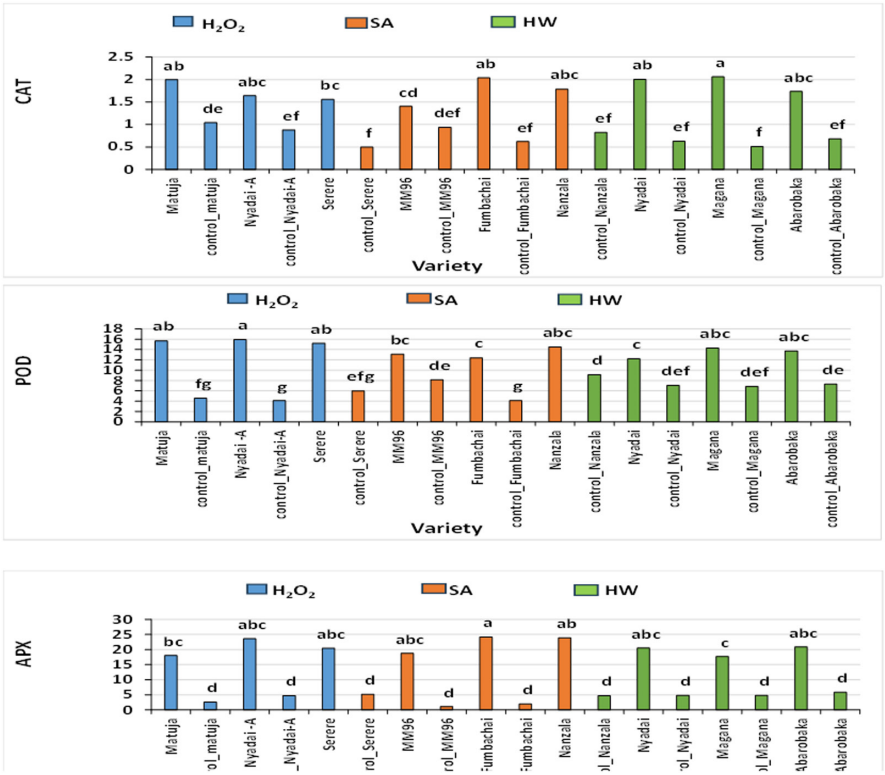

FIGURE 5 The activities of catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) (μmol g^{-1}FW) in the virus-eliminated plants obtained from SA, H_2O_2 and HW-treated stem cuttings and plants from the untreated control stem cuttings. The values are means of three biological replicates repeated three times (three technical replicates). Similar letters in the error bar show no significance.

in response to SA, H_2O_2 and HW treatments. There is also a need to determine the effect of different treatment combinations such as HW with SA in eradication of geminiviruses from infected cassava plants.

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 substantially reducing the viruses from planting material. Based on the varieties used in this study, HW treatments at 50°C 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 cassava stem cuttings. Based on biochemical assays conducted in parallel, it is hypothesized that the treatments prevented virus replication and halted the progression of the disease by suppressing production of H_2O_2, reducing lipid peroxidation and upregulating the activity of CAT, POD and APX enzymes. Applying and combining the different treatment methods presented in this study and understanding the factors contributing to the success of virus reduction holds significant potential to produce virus-free propagation material.

AUTHOR CONTRIBUTIONS

Mercy W. Kung'u: Data curation; Formal analysis; Investigation; Methodology Validation; Visualization; Writing – original draft. **Evans N. Nyaboga:** Conceptualization; Methodology; Data curation; Funding acquisition; Supervision; Writing – review & editing. **Chrissie Rey:** Conceptualization; Funding acquisition; Supervision; Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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