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## SOIL & CROP SCIENCES | RESEARCH ARTICLE

# Production of virus-free cassava through hot water therapy and two rounds of meristem tip culture

Grace Nakabonge<sup>1\*</sup>, R. Nangonzi<sup>2</sup>, B.S. Tumwebaze<sup>1</sup>, A. Kazibwe<sup>2</sup>, C Samukoya<sup>3</sup> and Y. Baguma<sup>4</sup>

**Abstract:** Cassava genetic resources are highly threatened by devastating viral diseases. It is therefore important to conserve the specifically farmer-preferred landraces/cultivars that are highly susceptible. The study assessed the efficiency of hot water therapy and meristem tip culture techniques in the elimination of cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) causal viruses. Stems of symptomatic cassava genotypes confirmed infected with cassava brown streak viruses (CBSV) and cassava mosaic viruses (CMV) were subjected to hot water therapy at varying temperatures (25°C—60°C) followed by two rounds of meristem tip culture (MTC). Data on sprouting in the screen house and response *in vitro* were recorded and weaned plants were indexed for CMV and CBSV. The results indicated 100% elimination of CMV and a significant reduction in the viral load of CBSV. Thus, these techniques could be integrated into the cassava conservation strategy, for sustainable management of cassava genetic resources.

**Subjects:** Agriculture & Environmental Sciences; Agriculture and Food; Microbiology; Biotechnology

**Keywords:** Cassava brown streak disease; cassava mosaic disease; *Manihot esculenta*; thermotherapy; cassava genetic resources

### ABOUT THE AUTHOR

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### PUBLIC INTEREST STATEMENT

Cassava, an important food crop in the tropics, is rapidly becoming an industrial crop capable of transforming livelihoods. However, devastating viral diseases are affecting its productivity. Using cheaper means of cleaning cassava planting material could promote continuous cultivation and thus conservation of its genetic resources. Thus, the techniques optimized during this study could be integrated into the cassava seed system and conservation strategy, for improved productivity and sustainable management of its genetic resources.

## 1. Introduction

Cassava (*Manihot esculenta* Crantz) is a staple food crop that is increasingly becoming important as an industrial raw material (FAO, 2013). For instance, its demand as a source of bio-ethanol is growing rapidly, currently ranking second to maize as a source of industrial starch (FAO, 2013). Thus, the crop has the capacity to transform household livelihoods, rural and national economies; however, cassava is challenged by viruses that are threatening both its productivity and genetic resources (Alicai et al., 2007; FAO, 2013; Legg et al., 2011). DNA begomoviruses; African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) and RNA Ipomoviruses; Cassava brown streak virus (CBSV) and Uganda Cassava brown streak virus (UCBSV) are the most prevalent in the region (Alicai et al., 2007; Alicai et al., 2016). These cause severe diseases ranging from leaf and stem distortion, diminished tuberous growth and total destruction of the fleshy edible calorie-containing root tissue (Alicai et al., 2007; Legg et al., 2011).

Crop genetic resources and their diversity are essential for successful genetic improvement for sustainable productivity of any crop, including cassava (Kawuki et al., 2016; Ng & Ng, 2002). Before the emergence of environmental stresses such as cassava viral diseases, farmers had been known to continuously maintain diversity on farm for various strategic reasons some of which are variety specific uses (Nakabonge et al., 2018). However, the adoption of new improved and disease-resistant genotypes is creating a risk for loss of cassava genetic diversity (Kawuki et al., 2016; Nakabonge et al., 2018) since farmers prefer recently introduced varieties. As a consequence, several farmers' preferred but susceptible genotypes are getting extinct due to their abandonment in favor of improved and tolerant varieties (Kawuki et al., 2016; Mbanzibwa et al., 2009; Mukiibi et al., 2019; Nakabonge et al., 2018). Although almost all landrace materials in farmers' fields/gardens are infected with CBSD and CMD, they are important for future breeding since they have other traits that have undergone farmer selection over time (Hershey & Debouck, 2010; Kawuki et al., 2016; Mukiibi et al., 2019). It is thus important to evaluate biotechnologies that can help in preserving the remaining cassava genotypes for sustainable and improved livelihoods and also ensure that new germplasm is generated over time.

Many attempts to clean cassava materials infected with CMV and CBSV using stem hot water therapy, chemotherapy, a combination of *in-vitro* thermotherapy and meristem tip culture techniques have been described (Maruthi et al., 2019; Mohammed et al., 2017; Mwangangi et al., 2014; P. Wasswa et al., 2010; Zinga et al., 2014). However, most of these methods are still expensive since they require the use of sophisticated equipment, thus the need for optimization of cheaper technologies that can sustain *in-vitro* conservation of cassava genetic resources.

Several studies have indicated that high temperatures used in thermotherapy affect plant-virus interactions with a tremendous reduction of virus-induced symptoms (Szittyta et al., 2003; Wang et al., 2018). On the other hand, meristem tips being actively dividing, and devoid of vascular tissues with high endogenous auxin and metabolic activity have little or no viruses (Pramesh & Baranwal, 2015; P. Wasswa et al., 2010; Wondimu et al., 2012). In the study conducted by P. Wasswa et al. (2010), thermotherapy using dry heat in combination with meristem culture technique provided a 40% elimination rate of cassava brown streak virus, providing hope for complete virus elimination through optimization of procedures across germplasm. The study by Zinga et al. (2014) using hot water treatment of cassava cuttings indicated that 40% of the cuttings treated had regenerated a plant with no CMD symptoms compared to 7% of untreated cuttings, confirming the effect of hot water therapy on virus activity, but were not able to show virus elimination by molecular tests. The current study was aimed at assessing the combined efficiency of hot water therapy with two rounds of meristem tip culture. It is envisaged that the protocol would allow the production of clean cassava, free from cassava mosaic and cassava brown streak viruses needed for *in-vitro* conservation of valuable landraces for which there is no clean field grown material. Additionally, the study would support the supply of certified planting materials to those who might need to cultivate them.

## 2. Materials and methods

### 2.1. Sample collection

Five farmer-preferred, best performing and commonly grown genotypes including two elites (NASE 2, TME 204) and three landraces (Alado Alado, Magana and Bao) (Table 1) were selected for the experiment. Infected leaves and stem sections showing clear CBSD and CMD symptoms were collected from the National Crops Resources Research Institute (NaCRRI) experimental field, located at Namuloge, 21 km from Kampala. For CBSV detection, two mature leaves were picked from the mother plant, wrapped in clean aluminium foil, labelled and immediately frozen. For CMV indexing, two young leaves from each plant were picked from the mother plant and stored in 5 ml, microcentrifuge tube (Sigma—Aldrich) with 1 ml of 70% ethanol. The tubes were then labelled and sealed with PARAFILM® (Sigma—Aldrich). Stem sections (8 cm) were cut (approximately 5 cm from ground level) from the same mother plants showing clear symptoms, wrapped in clearly labelled polyethylene gummy bags and transported to the laboratories at NaCRRI for further use.

### 2.2. Detection of cassava mosaic geminiviruses (CMGs)

A total of 15 samples (leaves) from the 5 cultivars were indexed for CMV. DNA was extracted following the cetyl trimethyl ammonium bromide (CTAB) method (Lodhi et al., 1994) as modified by (Ogwok et al., 2015). The quantity, purity and integrity of each extracted DNA sample were assessed using a NanoDrop (Model 2000 C; Thermal Scientific, USA). Conventional PCR was performed using ACMV-AL1/F, ACMV-ARO/R, UV-AL1/F, ACMV-CP/R3 primers (Zhou et al., 1997). ACMV-AL1/F/ARO/R was used for the detection of open reading frames (ORFs) AC1 and AV2 of ACMV; UV-AL1/F/ACMVCP/R3 for ORFs AC1 and AV1 specific for Ugandan EACMV strain EACMV-Ug (Ogbe et al., 2006). The PCR master mix components contained 10 µl of the PCR master mix (Premix, New England Biolabs), 0.2 µl of both F and R primers, 7.6 µl of sterile distilled water and 2.0 µl of the template DNA. Thermal cycling conditions were as described by (Ogwok et al., 2015). The PCR products were separated using 1.2% agarose gel electrophoresis using Galileo Minigel Systems (Galileo Biosciences, Massachusetts, USA) followed by UV documentation.

### 2.3. Detection and quantification of cassava brown streak viruses (CBSVs)

A total of 15 samples (Leaves) were indexed for CBSV. RNA was extracted following the CTAB extraction protocol as modified by (Ogwok et al., 2012) using approximately 100 mg leaf sample. Extracted nucleic acids were treated with DNase I according to manufacturer recommendations (Biolabs, Boston USA) to remove cassava genomic DNA. The quantity, purity and integrity of each extracted RNA sample were assessed using a Nano-Drop Spectrophotometer (Model 2000 C; Thermo Scientific, Waltham, USA). Quantitative RT-PCR was done to determine the CBSV levels (titer) in RNA samples with OD260/280 of 1.8–2.1. The samples were subjected to first-strand cDNA synthesis by RT-PCR using Revert Aid First Strand cDNA Synthesis Kit following manufacturer recommendations (Thermo Scientific, Waltham, USA). cDNA was subjected to rt-PCR following Maxima SYBR Green/ROX qPCR Master Mix (2X) SYBR Green I chemistry (Thermal Scientific). Cytochrome oxidase (COX) was used as an internal control with primers

**Table 1. Characteristics of selected cassava genotypes used in the study**

Stock ID	Genotypes	Pedigree	Source
CB 1498566	NASE 2	Introduction from IITA (International Institute of Tropical Agriculture)	National Agricultural Resources research Institute (NaCRRI), NARO, Uganda
UG110016	TME 204	Introduction from IITA	“
GN14	Alado Alado	Ugandan local variety	“
GN8	Magana	Ugandan local variety	“
UG110050	Bao	Ugandan local variety	“

COX-F (5'-CGTCGCATTCCAGATTATCCA-3'), COXR (5'-CAACTACGGATATATAAGRRCRRAACTG-3') in conjunction with other primer sets that is; UCBSVqF (5'-AAGGCAAGGGTGGCTCTAAC-3'), UCBSVqR (5'-GCGTCCTTGTTGGCCCATTA-3'), CBSVqF (5'-GCCAACTARAAGTCCATT-3'), CBSVqR (5'-TTCAGTTGTTAAGCAGTTCGTTCA-3') and probe (5'- [FAM]-AGGGCATTCCATCCAGCGTAAGCA-[TAMRA]-3') (Ogwok et al., 2015). For each RNA sample, two technical replicate reactions were prepared containing 12.5 µl of Maxima Probe qPCR Master Mix (2X) (Fermentas, Ontario Canada), 7.5 µM of each forward and reverse primer, SYBR Green mix, 100 ng of template and nuclease-free sterile water to volume of 25 µl. In addition, non-template water control was included on every plate. The reactions were incubated for 60 min at 42°C then initial denaturation step run for 10 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 30 sec at 60°C and extension for 30 sec at 72°C.

Reverse transcriptase PCR was performed on an Applied Biosystems, One Step Plus® sequence detection system (Applied Biosystems, Waltham, USA) using Bio-Rad CFX96 Connect qRT-PCR, equipped with the CFXMANAGER software for data analysis (Bio-Rad Laboratories Inc., Hercules USA) as described by Ogwok et al. (2015). Results for the reduction of CBSV levels were displayed as an output on the instrument monitor. The generated cycle threshold (C<sub>t</sub>) values were used to determine the fold change in expression of the target gene for both CBSV and UCBSV at the end of the experiment. All genotypes that had C<sub>t</sub> value of equal to or less than 40 for UCBSV or CBSV were considered to be free of the viruses.

#### **2.4. Hot water treatment of cassava stem cuttings**

Stem cuttings (8 cm and with approximately 4–5 nodes and 18 per cultivar) of positively confirmed CBSV and CMV leaf samples were subjected to thermotherapy treatments by incubation in a preheated water bath at varying temperature ranges (control (room temp of 25 °C), 40°C, 45°C, 50°C for 30 min and then 55°C and 60°C for 20 mins). The control treatment was not incubated. All heat-treated and non-treated cuttings were then planted in 90 plastic buckets in a completely randomized design with each treatment having a total number of 15 stakes replicated three times. Plant shoot sprouting in terms of shoot numbers and shoot height and disease development in terms of incidence and severity were assessed weekly for a period of 2 months.

#### **2.5. In-vitro propagation by meristem tip culture (MTC) and second-round/double meristem culture (DMTC)**

Regenerated plantlets were introduced *in-vitro* by meristem tip culture on Murashige and Skoog (MS) optimized basal medium (Murashige & Skoog, 1962). The media was prepared by weighing and adding individual media components; 4.4 g/l MS salts with vitamins, 20 g/l sucrose, 7.0 g/l agar and 0.5 mg/l BAP in 500 ml of distilled water. The components were homogeneously mixed with a magnetic stirrer. The solution was then topped up to 1 l with distilled water and pH adjusted to 5.8 using 2 M HCl and 1M NaOH. The semisolid medium was prepared by the addition of 7.0 g/l agar and autoclaving at 121°C for 15 min. This medium was allowed to cool to about 70°C and then dispensed into sterile petri dishes, cooled and sealed for further use.

Shoot tip cuttings of about 4 cm (with 2 to 3 nodes) in length were collected from the five hot water treated and regenerated cassava cultivars wrapped in labelled aluminum foil and transferred to the tissue culture laboratory. The shoot tips as explant material were subjected to two rounds of sterilization. The explants were washed with water and liquid soap 10% Sodium Alkylbenzene sulfonate (Sameg Chemical Products Uganda Ltd.) by gentle shaking. Water with detergent was poured off and explants rinsed 4 times with tap water. 70% ethanol was added and left to stand for 1 min. Ethanol was then poured off and 2 ml water with 2–3 drops of Tween-20 and 0.5% NaOCl (Jik) was added and the tube had shaken gently. After 5 min, tissues were rinsed 4–5 times using sterile water. The apical dome with one or two leaf primordia of 0.3 to 0.5 mm in size was excised under a zoom binocular dissecting microscope (Nikon metrology, SMZ445160; stereo microscope 360 × 308) and immediately inoculated onto solid MS initiation media. Labeled media plates were immediately sealed with both PARAFILM® (Sigma—Aldrich) and cling film to prevent desiccation and contamination. The cultures were then incubated in a growth room with a temperature regime of 24°C ± 1°C under 16-h photoperiod provided by fluorescent bulbs with

a light intensity of 1500 lx, for 6 weeks. The plates were arranged in batches according to temperature treatment. Each treatment had a total of 10 plates (2 samples per genotype). Thereafter, meristems were transferred to fresh normal semi-solid MS media for multiplication, shoot elongation and root formation for a period of four weeks. After 4 weeks, another round of initiation (DMTC) was done as described previously.

### 2.6. Plant establishment (weaning)

Rooted shoots from the laboratory (*in-vitro* cultures) were weaned on a 4:1 v/v mixture of sterile soil and vermiculite. These were visually monitored weekly for survival and appearance of either CBSD or CMD symptoms for a period of six weeks. Lower leaf samples were collected from weaned plantlets and indexed for CMV and CBSV as previously described.

### 2.7. Data analysis

Shoot regeneration following hot water therapy was evaluated in terms of shoot number, shoot height and number of leaves per shoot. Descriptive statistics such as mean and standard deviation were used to summarize the data. Analysis of variance (ANOVA) using the SPSS v23.0 software was used to assess the effect of hot water treatment on the shoot regeneration parameters under *in-vitro* and screen house conditions. The combined effect of both hot water treatment and double meristem tip culture for CMV and CBSV elimination was determined as the proportion of CMV negative samples after PCR confirmation and CBSV titer after qPCR.

## 3. Results

### 3.1. Initial detection of CMV and CBSV in collected samples before treatment

UCBSV with 437 bps band was the only strain of CBSV detected from the samples (Bao, Magana, Alado, TME 204 and NASE 2) used in the study. Real-time quantification of UCBSV revealed TME 204 (sample 5) having the least relative viral load in folds ( $\Delta\Delta Ct$ ) and TME 204 (sample 4) having the highest load (Table 2). Mixed infections of ACMV and EACMV-Ug with 1000 and 1500 bp bands, respectively, were

**Table 2. Relative virus titre indicated by qPCR. CT values in symptomatic leaves of cassava genotypes (1–3 Magana, 4–6 TME 204, 7–9 Bao, 10–12 Alado, 13–15 NASE 2) infected with UCBSV**

Sample no.	Cox	UCBSV	CBSV
1	40	40	40
2	26.2398	38.3729	40
3	29.4145	38.1553	40
4	27.6625	20.8015	40
5	31.1989	40	40
6	31.8789	37.6626	40
7	29.3971	32.3251	40
8	19.338	38.2058	40
9	18.2094	36.7018	40
10	17.7041	35.6388	40
11	18.9127	35.4081	40
12	18.7388	36.2124	40
13	20.3409	35.1685	40
14	19.802	35.2897	40
15	20.6679	38.7062	40
NTC	40	40	40
PTC	24.2875	19.9291	40

detected in Magana (lanes; 1, 2, 3), Bao (lanes; 7, 8, 9), Alado (lanes; 10, 11, 12) and NASE 2 (lanes; 13, 14, 15) (Figure 1). Samples of TME 204 (lanes; 4, 5 and 6) that tested negative for CMV tested positive for UCBSV. Co-infections with both CBSV and CMV were detected in 87% of the collected samples while mixed infections were detected in 73.3% of the CMV diseased samples.

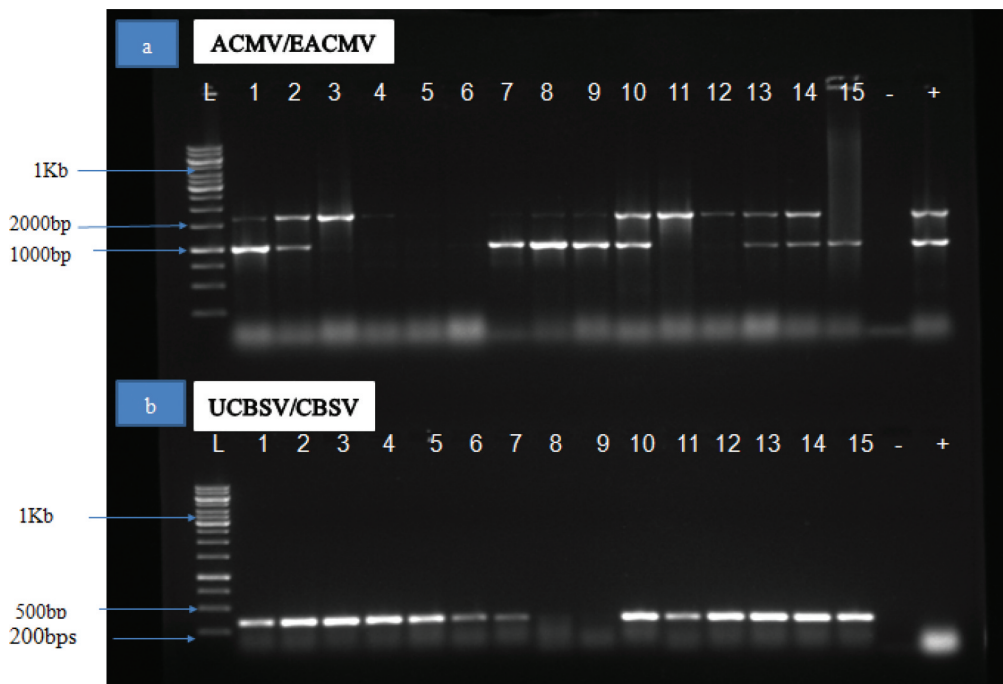
$C_t$  values (Generated cycle threshold) for the initial quantification of the viruses where the lower the  $C_t$  value, the higher the virus titer and vice versa. COX (cytochrome oxidase) the reference gene, NTC (Non-template water) the negative control and PTC positive control (sample known to be positive for UCBSV).

### 3.2. Sprouting following hot water treatment

Analysis of variance indicated significant interactions ( $P < 0.00$ ) (Table 3) between temperature treatment and type of cultivar on the number of shoots. Bao recorded its maximum shoot number at control temperature (25°C), Alado at 40°C, Magana at 45°C and TME 204 and NASE 2 at 55°C (Figure 2).

Shoot length was significantly ( $P < 0.01$ ) influenced by the interactions between cultivar, treatment and time (Table 3). However, there was variation in length among the cultivars across all temperature treatments. TME 204 recorded the highest mean length of shoot, followed by Magana, Alado, Bao and NASE 2 recorded the least mean value in shoot length.

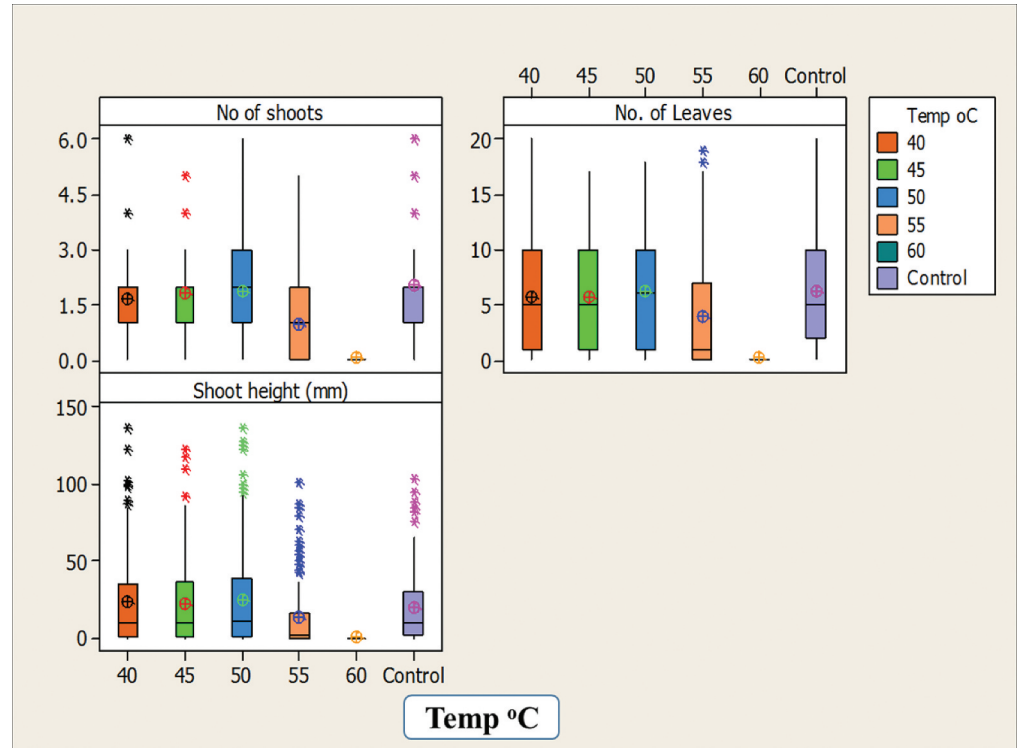
**Figure 1. Detection of (a) ACMV and EACMV-Ug and (b) UCBSV in a set of field-collected symptomatic leaf samples; where 1–15 represent the samples, L represents DNA ladder of known size in Kilobases (1kb),— is the negative control (containing nuclease-free water).**



**Table 3. Analysis of variance (ANOVA) of response of four cassava cultivars to hot water therapy in terms of number of leaves and shoots and shoot height (mm) under various temperatures (40°C, 45°C, 50°C, 55°C, 60°C)**

Response	F	P	R-Sq (%)	StDev
Number of leaves	30.55	0.000	17.63	4.641
Number of shoots	55.23	0.000	27.89	1.113
Shoot height (mm)	15.58	0.000	9.84	25.63

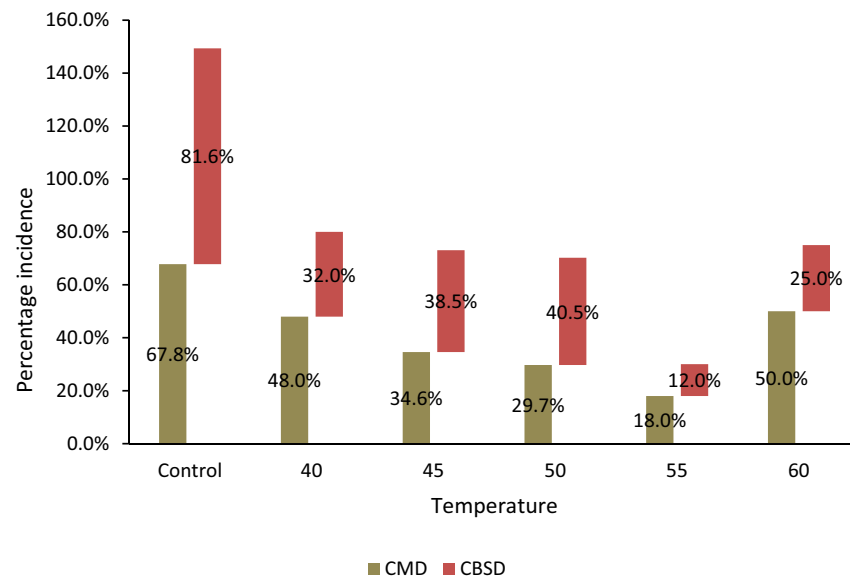
**Figure 2. Variation in; number of shoots, shoot heights and number of leaves following exposure of cassava cultivars to five temperature treatments.**



### 3.3. Disease incidence

Symptom development for both diseases generally decreased with increase in temperature treatment. Lower temperatures such as in the control and 40°C recorded 100% disease incidence and higher temperatures, 55°C (20%) and 60°C (6.7%), recorded the lowest percentage of disease incidence. However, no temperature treatment recorded zero percent disease incidences for both CBSD and CMD (Figure 3). The predominance of both disease symptoms also significantly varied among the selected cultivars, Alado and Bao showed the highest incidence of both diseases followed by Magana.

**Figure 3. Percentage incidence of CMD and CBSD on cassava cuttings under the different temperature treatments; including control (25 °C), 40°C, 45°C, 50°C, 55°C and 60°C.**





### 3.4. *In-vitro* propagation by meristem tip culture (MTC) and second round of meristem tip culture (DMTC)

Treated and control plants were successfully introduced *in vitro* in varying percentages that is 86.6%, 92%, 84.6%, 64.3%, 81.8% and 100% at control, 40°C, 45°C, 50°C, 55°C and 60°C, respectively, during the first round of meristem culture. During the second round of meristem tip culture, there was no significant difference ( $P < 0.005$ ) in overall response (regeneration) among the different varieties in terms of number of leaves, stem height and root numbers. In general, the number of leaves, shoot height and root numbers were observed to increase over time.

### 3.5. Plant establishment (weaning)

The percentage survival of weaned MTC plantlets was 90.3%, 84.1%, 83.5%, 80.8% and 80.7% in cultivars; Magana, Alado, Bao, NASE 2 and TME 204, respectively. Alado had the poorest growth vigor on weaning compared to other genotypes evidenced by stunted growth, leaf mottling, reduced leaf size and early withering of young leaves. Plant survival rate in DMTC plantlets was better than that in MTC plants. 100% survival was recorded in all the DMTC weaned plantlets. Generally, very low disease incidence (7.14%) was observed in DMTC plantlets compared to MTC weaned plantlets (Figure 4).

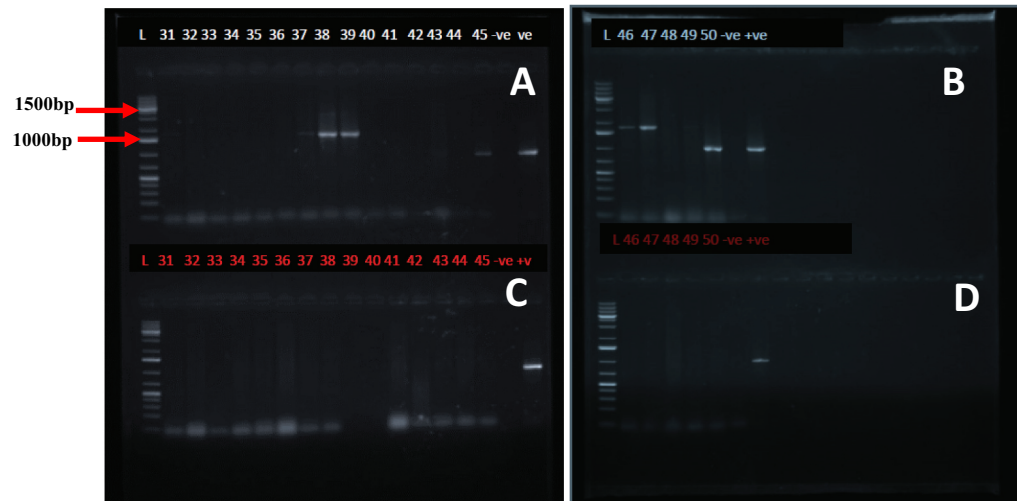
### 3.6. Molecular detection of CMV and CBSV in plant samples treated by a combination of Hot water treatment, MTC and DMTC

CMV positive samples were detected in plantlets treated at lower temperature treatments (i.e. 40°C) and CMV negative samples treated at higher temperatures of 55°C and 60°C following MTC. Analysis of samples that were subjected to DMTC did not reveal any cassava mosaic viruses, this was also true for the control (not hot water treated plants) (Figure 5) and RT-qPCR detected very low titer loads of CBSV (UCBSV) (Figure 6). The results clearly show that MTC had reduced the UCBSV virus titer from the initial Ct 20.8 (Table 2) for the sample with the highest titer to Ct 17.

**Figure 4.** Symptom development of cassava genotypes upon weaning prior to subjection to hot water treatment at various temperatures and meristem tip culture. Variation in disease symptoms at various temperatures (A & B) and response at MTC (C) and DMTC (D).



**Figure 5. Gel electrophoresis results for detection of ACMV and EACMV-Ug viruses in samples subjected to hot water therapy followed by MTC (meristem tip culture, gel A and B) and DMTC (second round of meristem tip culture, gel C and D).**



#### 4. Discussion

Results from the present study indicate that novel technologies such as application of a combination of hot water therapy and two rounds of meristem tip culture (Figure 9) completely eliminated CMV and significantly reduced the viral load of UCBSV from infected cassava genotypes.

##### 4.1. Initial detection of CMV and CBSV

Conventional PCR and RT-PCR confirmed the presence of the target viruses by producing clearly distinct bands of co-infected, mixed and single infections of UCBSV and CMV. This indicated that the selected cultivars are susceptible to either one or both species and strains of the target viruses. The presence of higher virus titers of UCBSV in TME 204 and in the local cultivars Bao, Magana and Alado confirms their high susceptibility to CBSV infections (Ogwok et al., 2015). All indexed samples tested positive for mixed infections of both CMV and UCBSV except 3 samples of TME 204 (4, 5 and 6) that tested negative for both ACMV and EACMV but were positive for UCBSV. The sensitivities of the used detection tools to clearly detect and distinguish the two specific viruses causing CBSD and CMD confirm their sensitivity and suitability in the diagnosis of cassava viral diseases as previously reported (Aloyce et al., 2013).

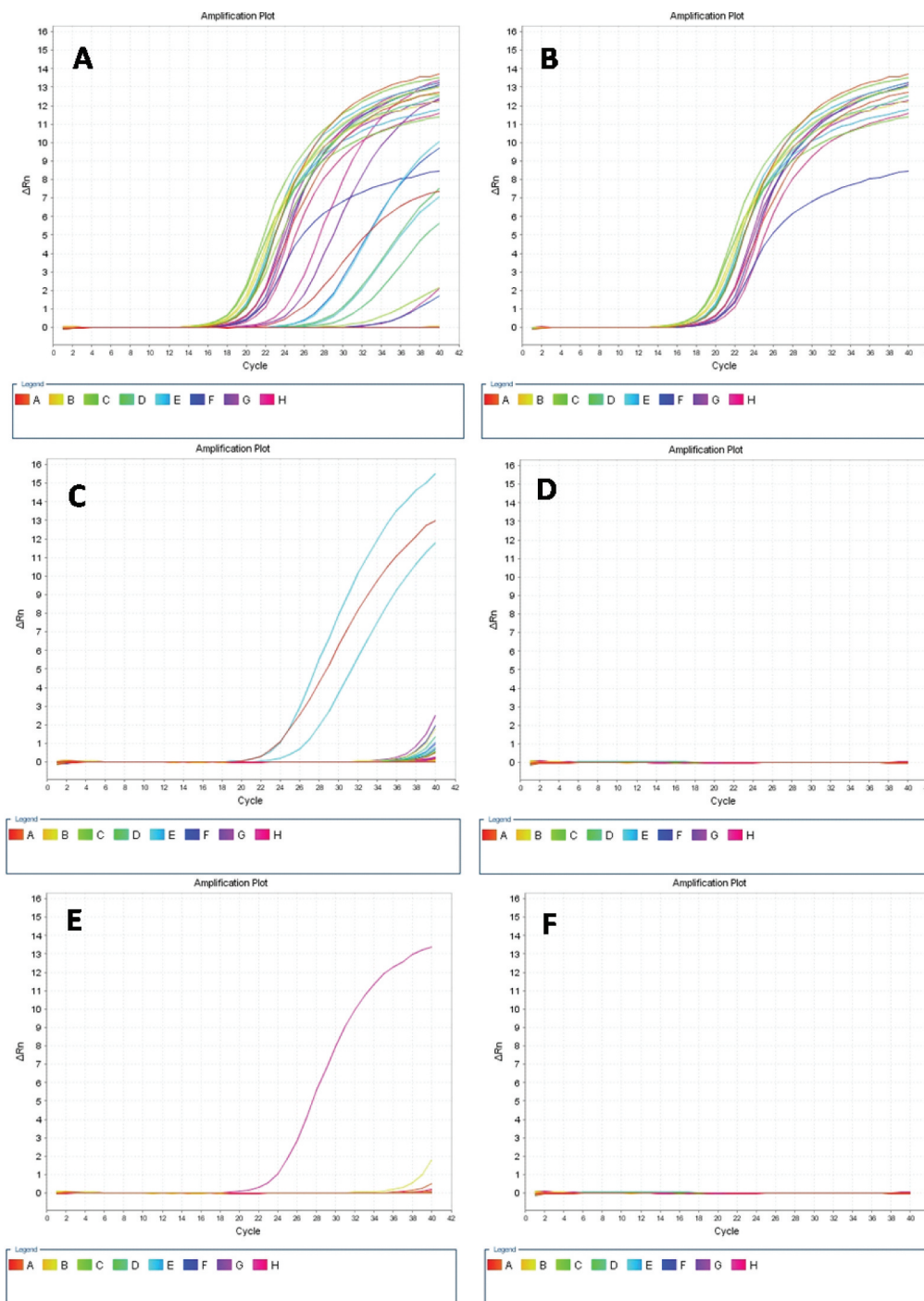
##### 4.2. Survival and shoot regeneration following hot water treatment

Generally, a high percentage (87%) of cassava plant survival was observed following hot water therapy. However, the survival among treated plants significantly decreased with the increase in temperature across all varieties. Greater than 80% survival was observed at lower temperatures below 45°C. The lethal effect of heat at higher temperatures is consistent with studies by Mohammed et al. (2017) and Kidulile et al. (2018) who observed low plantlet survival to complete death of plants at temperatures higher than 45°C in *in-vitro* thermotherapy chambers. Previous studies on hot water therapy for pathogen elimination from infected plants had reported a physiological temperature tolerance limit of 35° and 54° for most plants (Grondeau et al., 1994; Panattoni et al., 2013) as being the compromise between virus degradation and plant survival. In the current study, optimal survival was observed with incubation at 50°C which was within the range of other previous studies.

##### 4.3. The *in-vitro* response of heat-treated selected cassava genotypes following meristem tip culture

*In-vitro* response on both initiation and normal media was significantly ( $P < 0.01$ ) influenced by the selected cultivars. This confirms differential cultivar sensitivity for meristem culture and regeneration as reported previously by P. Wasswa et al. (2010). The number of leaves in the best responding

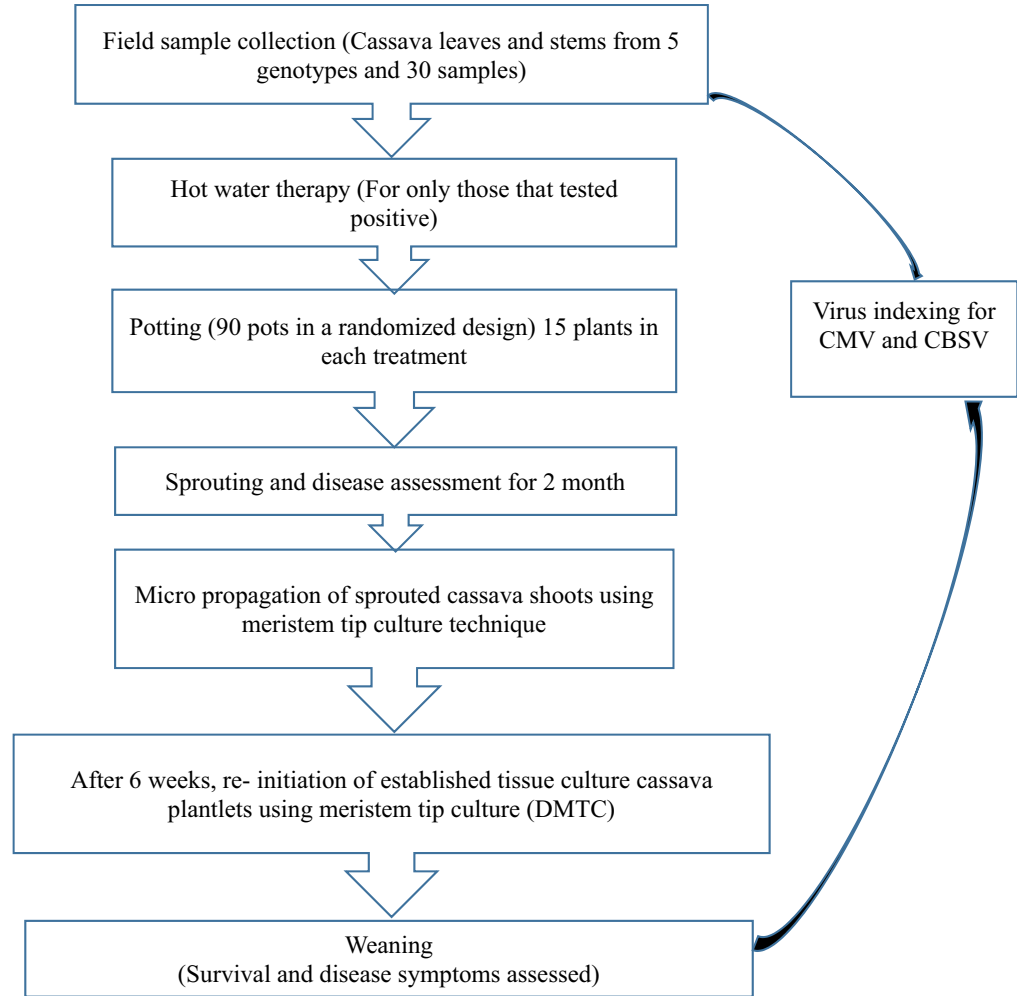
**Figure 6. qPCR amplification plots and standard curves of UCBSV (A) samples before treatment, (B) COX, the house keeping gene, (C) Amplification of UCBSV following MTC (D) Amplification of CBSV in the MTC; (e) Amplification of UCBSV following DMTC (F) Amplification of CBSV following DMTC.**



genotypes correlated with the number of roots, suggesting that the higher number of roots provided a large surface area for absorption of nutrients from the media (Patial et al., 2012).

The size of the meristematic dome cutting has been reported as an important factor in the regeneration of shoots under *in-vitro* laboratory conditions. The effect of meristem size on regeneration was not investigated in this study; however, Mwangangi et al. (2014) reported a higher number of plants regenerating from larger meristems of 1 mm and 2 mm than in smaller meristem tip size of 0.5 mm. In this study, the excision of a small meristematic dome of ~0.5 mm from regenerated shoots

**Figure 7. Schematic diagram of hot water therapy and 2 rounds of meristem tip culture to generate CMV and CBSV free plants.**



of the first meristem culture resulted in 100% regeneration of shoots with 100% CMV elimination and a considerable reduction (97.7%) of CBSV in plants treated at 55°C and 60°C. Therefore, small meristems can result in high regeneration and higher virus elimination as compared to larger meristems.

#### **4.4. Combined efficiency of hot water therapy, first and second round of MTC**

Meristem shoot tip culture, hot water therapy or a combination of both methods have been applied to obtain cassava plants free from cassava mosaic and cassava brown streak viruses (P. Wasswa et al., 2010). Results of this study are in agreement with previous reports that hot water therapy can reduce the level of CMV (Zinga et al., 2012) and meristem tip or a combination of both can either eliminate CMV or reduce CBSV (Mwangangi et al., 2014; Wang et al., 2006; P. Wasswa et al., 2010; M. Wasswa et al., 2017; Zinga et al., 2012). However, the study by Zinga et al. (2012) used hot water therapy alone, whereas that of P. Wasswa et al. (2010, 2017) used thermochambers with dry heat. The current study is the first of its kind to combine hot water therapy and rounds of meristem tip culture for the elimination of both CMV and CBSV.

Due to limited time and resources, the study included a few samples; follow-up studies should consider more cassava varieties and explore the use of more than two rounds of meristem tip culture for the complete elimination of CBSV.

In conclusion, we successfully generated CMV-free plants from samples treated at 55°C combined with two rounds of meristem tip culture. Furthermore, the same protocol tremendously reduced the virus load in UCBSV-infected samples. Therefore, stem hot water therapy followed by at least two or three rounds of meristem tissue culture can generate virus-free cassava plants and thus could be recommended for use in the *in-vitro* conservation and use of cassava virus-free genetic resources.

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#### Competing interest

Authors declare no competing interest.

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