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RESEARCH ARTICLE

Quantitative evidence on reduction of CMD virus inoculum as influenced by management practices in cassava

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Abstract

Cassava mosaic disease (CMD) poses a substantial challenge to the success of cassava cultivation in India, primarily attributed to the *Indian cassava mosaic virus* (ICMV) and the *Sri Lankan cassava mosaic virus* (SLCMV). This study examines the impact of diverse management techniques on diminishing CMD viral load in cassava plants, employing quantitative PCR (qPCR) as the principal measurement instrument. The experiment was conducted in a cassava field, with treatment plots assigned in a randomized block design (RBD). The research evaluated the effectiveness of different nutrient applications and insecticidal treatments. Results indicated that treatments, such as fish oil rosin soap and cassava booster, significantly reduced CMD viral concentrations 14 days post-application. The qPCR results showed a substantial decrease in virus copy numbers following these treatments. The study highlights the importance of integrated disease management strategies for CMD control, demonstrating how these practices can lead to healthier cassava crops, increased yields and sustainable crop protection.

Keywords

cassava mosaic disease; deoxyribonucleic acid (DNA) reduction; quantitative polymerase chain reaction (qPCR); viral load

Introduction

Cassava (*Manihot esculenta* Crantz), one of the world's major staple crops, is grown in India for both food and industrial purposes. As a tropical crop, cassava plays a crucial role in food security and is a source of income for many low-income farmers in developing countries. Fresh cassava tubers serve as an essential source of calories for over a billion people across approximately 105 countries, benefiting both human and animal consumption. Cassava starch is also widely used in industries such as bioethanol production, paper manufacturing, animal feed and as a thickening agent in food processing. Cassava's resilience to adverse conditions and abiotic stress makes it an ideal crop for resource-limited, small-scale farmers, outperforming cereals like wheat, rice and maize in challenging environments (1). In India, cassava production is estimated at 6.2 million tonnes (Mt) annually (2).

Cassava production, however, faces several challenges. Notably, CMD, caused by eleven species of cassava mosaic viruses collectively known as cassava mosaic begomoviruses (CMBs), poses a significant threat to cultivation. CMD primarily spreads through infected planting materials, with a secondary

transmission mode via the whitefly (*Bemisia tabaci Gennadius*). Research has confirmed that CMD is not seed-transmissible (3) but can be effectively transmitted through grafting techniques (4). The biological characteristics of *B. tabaci* such as its multivoltine nature, high reproductive capacity, wide host range and long-distance migration-complicate the development of sustainable management strategies for this pest. Cassava plants begin producing leaves within 2-3 weeks of planting, and it is during this early stage that young leaves are colonized by viruliferous whiteflies (5). This period is critical for CMD geminivirus infection, as older plants are not susceptible (6). Adult whiteflies can continue to infect healthy plants for up to 48 hours after virus acquisition, with transmission likelihood increasing when multiple infected whiteflies feed on a plant (7). The coat protein (CP) of geminiviruses is adapted to local *B. tabaci* populations, resulting in antigenic similarity among CPs of regional begomoviruses (8).

Cassava mosaic disease, transmitted by *B. tabaci*, has led to yield losses ranging from 44% to 90% in Africa and from 10% to 88% in India (9-12). Studies have reported an 80.5% transmission efficiency of the virus via *B. tabaci* over three months from cassava to cassava (13). Disease severity can result in yield losses of 20 % to 90 % (14).

In the Indian subcontinent, CMD was first reported in 1956 and further described in 1966 (15, 16). The two main viral species causing CMD in India are the *Indian cassava mosaic virus* (ICMV) and the SLCMV, with SLCMV being the most widely distributed and prevalent in Tamil Nadu (17).

The severity of CMD can be assessed through visual examinations and quantified using real-time PCR (RT-PCR) or qPCR analysis. Infected plants show symptoms such as leaf deformation, stunted growth, reduced root and tuber production (without root rotting) and irregular yellow or yellow-green chlorotic mosaic patterns on the leaves (18-20). Quantitative PCR is a reliable and advanced method for diagnosing CMD. This technique combines PCR with fluorescent reporter chemistry, allowing for real-time tracking of template amplification with enhanced sensitivity and specificity. qPCR is widely used for the absolute quantification of gene expression, counting microorganisms or copy numbers and studying relative gene expression. This method monitors the increase in fluorescence from the reporter molecule during the exponential phase of template amplification. Two types of assays exist for qPCR: probe-based and dye-based. SYBR Green is a commonly used dye-based chemistry, where the dye intercalates with double-stranded DNA during the primer extension step performed by the polymerase (21).

Cassava mosaic disease management can be achieved using resistant and tolerant cultivars as planting material. Alternatively, nutrient-based management strategies and vector control methods can be implemented to combat the disease under field conditions. In this study, the effectiveness of various management methods in reducing CMD viral load was evaluated using qPCR.

Materials and Methods

Devising Management modules

An experiment was conducted on a farmer's field in Kallakurichi district, Tamil Nadu, where the susceptible cassava cultivar "White Thailand" is widely cultivated. Treatment plots in the experimental field were randomly assigned using a random number table. Weather parameters recorded during the survey period included a maximum temperature of 40.86˚C, minimum temperature of 24.86˚C, humidity at 51.37%, rainfall of 1.17 mm, and 7.32 hours of sunshine. The experiment was set up using a RBD with three replications for each of the treatments. The treatment details adopted in the experimental setup are illustrated in Table 1.

Table 1. Treatment details adopted in the experimental setup

Treatment Code	Treatment details
T1	Spraying cassava booster at 5kg/acre, 60 days after planting (DAP)
T2	Spraying azadirachtin 0.03% at 3 mL/L, 60 DAP
T3	Spraying fish oil rosin soap at 25g/L, 60 DAP
T4	Spraying thiamethoxam 25G 0.5 g/L, 60 DAP
T5	Using 5 yellow sticky traps/acre + Spraying cassava booster at 5kg/acre, 60 DAP
T6	Using 5 yellow sticky traps/acre + Spraying cassava booster at 5kg/acre + Spraying azadirachtin 0.03% at 3 mL/L, 60 DAP
T7	Using 5 yellow sticky traps/acre + Spraying cassava booster at 5kg/acre + Spraying fish oil rosin soap at 25g/L, 60 DAP
T8	Using 5 yellow sticky traps/acre + Spraying cassava booster at 5kg/acre + Spraying thiamethoxam 25G at 0.5 g/L, 60 DAP
T9	Control (treatment)

Sample collection

Leaf samples were obtained from all treatment plots over three replications, both prior to and subsequent to the application of a treatment and were appropriately labeled. Five young apical leaves were taken from the uppermost shoot for examination. Approximately 100 mg of both symptomatic and non-symptomatic leaf samples were gathered from newly emerging leaves. Treatment (T1 to T8) was then sprayed on the assigned plots in the trial field, while T9 served as the untreated control plot**.** Samples were collected at 0, 7 and 14 DAS, as virus incidence tends to increase during the vegetative phase when cassava exhibits vigorous growth between 60 to 90 DAP (5). Accordingly, treatments were applied at 60 DAP. Leaf samples from the top young leaves were collected at 0, 7 and 14 DAS and were separately covered and preserved for further analysis.

DNA extraction procedure

DNA extraction from the collected leaf samples followed the cetyltrimethylammonium bromide (CTAB) method (22). Each 100 mg of leaf sample was finely ground using liquid nitrogen. To the resulting powder, 1 mL of prewarmed CTAB buffer was added and the sample was transferred to a 2 mL centrifuge tube. The tube was incubated for 30 min at 60˚C and then centrifuged at 10,000 rpm for 10 minutes at 27˚C. Following this, 1 mL of chloroform isoamyl alcohol mixture (24:1) was added to the supernatant. The mixture was centrifuged again

at 13,000 rpm for 10 minutes at 4˚C. The aqueous layer was transferred to a new 1.5 mL tube and an equal volume of ice-cold isopropanol was added, followed by incubation at -20˚C for 1 hour. Afterward, the mixture was centrifuged at 13,000 rpm for 15 minutes at 4˚C and the supernatant was discarded. The pellet was washed with 70% ethanol and after discarding the ethanol, the pellets were air-dried. Finally, the pellet was re-suspended in 20µL of nuclease-free water. This process was repeated for all 81 samples, and the collected DNA pellets were subsequently analyzed by qPCR.

Quantitative PCR (qPCR)

The qPCR analysis was performed on the extracted DNA from pre-treatment samples and samples collected 7 and 14 DAS, employing the BioRad CFX Opus 96 System. In this study, an absolute quantification standard was employed, utilizing SYBR Green dye as the fluorophore. Dye-based qPCR enables real-time monitoring of DNA amplification by using a fluorescent dye, such as SYBR® Green, which binds to double-stranded DNA (dsDNA). Serially diluted samples were prepared down to a concentration of 100 ng. A plasmid vector containing an insert of the sequence of the TVM3 cassava mosaic virus isolate, maintained in the CTCRI lab, was used to prepare the standard following the alkaline lysis method (23). To quantify the copy numbers of DNA-A and DNA-B molecules, qPCR was calibrated with controlled samples containing standard concentrations of plasmids carrying full-length copies of the DNA-A and DNA-B components, respectively. The molecular weight of each plasmid was calculated based on the sizes of the plasmid and viral constructs and serial dilutions of plasmid DNA were prepared to create standard curves, which were then used to calculate the copy numbers of the genomic components. The concentration of the isolated plasmid DNA was 70.9 ng/µL, measured using a Denovix DS 11+ spectrophotometer and this value was used to determine the copy number of the plasmid with the following formula (24).

$$\text{Plasmid copy number (copies/µl)} x = \frac{\text{Plasmid concentration (g/ µl)} \times 6.022\times10^{23}}{\text{Total fragment length (bp)} \times 660\ \text{(g/mol)}} \quad \text{(Eqn. 1)}$$

$$\text{Total fragment length} = \text{Vector length (bp)} + \text{fragment length (bp)} \quad \text{(Eqn. 2)}$$

The qPCR reaction was conducted using a 96-well plate (BioRad, Germany), with a total reaction volume of 10 µL per well. The composition of the reaction mixture is detailed below (Table 2).

Table 2. Composition of the reaction mixture for qPCR

Components	Volume/ Quantity
BioRad Sso Advanced Universal SYBR Green super mix	5 µL
Forward primer	0.25 µL
Reverse primer	0.25 µL
DNA template	100 ng
Nuclease free water	3.5 µL
Total volume	10 µL

The first 15 wells of the plate were loaded in duplicate with a set of five serially diluted standards. The unknown samples, positive control, and non-template control (NTC) were then added in triplicate. The unknown samples included pre-treatment and treated samples collected at intervals of 7 and 14 DAS. The plate was sealed with a plate sealing film and placed inside the thermal cycler. The cycling configurations included: initial denaturation at 95˚C for 10 minutes, denaturation at 95˚C for 15 seconds and annealing at 60˚C for 1 minute, repeated for a total of 40 cycles. After this program, a melt curve was generated, capturing fluorescence continuously from 65˚C to 95˚C with a 0.5˚C increment per minute. The CFX Maestro software was used to set up the thermal profile and plate parameters. The results provided quantification cycle (Cq) or cycle threshold (Ct) values for each well, with the average of triplicate readings considered valid. A difference of less than one cycle between at least two of the triplicate readings was expected to ensure reliable results.

Results

Effect of different treatments and their combination on virus titer

This study investigated the effects of various chemical treatments and their combinations on the susceptible cultivar, White Thailand. Among the nine treatments tested, two treatments demonstrated the most promising results (Table 3). A Standard curve was used to compare the unknown samples to standards with known DNA concentration, allowing for the estimation of the DNA concentration of the unknown samples (Fig. 1 and Fig. 2).

The application of cassava boosters (T1) effectively reduced viral concentration during the observation period. Initially, the virus copy number was 3.71×10^6, which decreased to 1.32×10^6 and 1.16×10^5 at 7 and 14 DAS, respectively. Plants treated with 0.03% azadirachtin (T2) showed an increase in viral load, with the copy number rising from 2.24×10^5 to 4.16×10^5 at 7 DAS, then decreasing to 5.95×10^3 at 14 DAS after treatment. Similarly, plants treated with fish oil rosin soap (T3) had an initial viral load of 2.44×10^5, which decreased to 8.36×10^4 and 8.34×10^3 at 7 and 14 DAS, respectively.

Treatment with thiamethoxam 25G (T4) initially had a swift impact, decreasing viral load at one week; nevertheless, the viral concentration subsequently escalated. The use of yellow sticky traps combined with cassava booster (T5) reduced viral load from 3.23×10^6 to 3.23×10^6 at 7 DAS and further to 1.56×10^5 at 14 DAS. The combination of cassava booster, azadirachtin and yellow sticky traps (T6) decreased the viral load from 3.50×10^6 to7.85×10^5 at 7 DAS and further to 9.11×10^4 at 14 DAS. However, the combination of cassava booster, fish oil rosin soap and yellow sticky traps (T7) lowered the viral load from 3.40×10^6 to 1.73×10^3 after one week, but the viral load increased again to 2.05×10^5 in the second week. In plants treated with cassava booster and thiamethoxam 25G along with yellow sticky traps (T8), the viral load decreased from 3.37×10^7 to 1.49×10^6 at 7 DAS and further to 8.84×10^5 at 14 DAS.

Table 3. Efficacy of different treatments against SLCMV in pre and post-treated samples

Treatments No.	Treatments	Pre-treatment		7 DAS		14 DAS	
		Mean Cq	Viral load	Mean Cq	Viral load	Mean Cq	Viral load
T1	Cassava booster*	11.77	3.71×10^{6}	12.54	1.32×10^{6}	13.79	1.16×10^{5}
T2	Azadirachtin 0.03%	12.89	2.24×10^{5}	19.90	4.16×10^{5}	25.08	5.95×10^{3}
T3	Fish oil rosin soap	13.16	2.44×10^{5}	18.76	8.36×10^{4}	20.93	8.34×10^{3}
T4	Thiamethoxam 25G	10.88	2.74×10^{6}	15.60	3.57×10^{5}	14.31	9.84×10^{5}
T5	Yellow sticky trap + Cassava booster*	11.79	3.23×10^{6}	13.88	2.25×10^{5}	14.50	1.56×10^{5}
T6	Yellow sticky trap + Cassava booster* + Azadirachtin 0.03%	12.19	3.50×10^{6}	14.22	7.85×10^{5}	14.70	9.11×10^{4}
T7	Yellow sticky trap + Cassava booster* + Fish oil rosin soap	11.67	3.40×10^{6}	23.25	1.73×10^{3}	17.77	2.05×10^{5}
T8	Yellow sticky trap + Cassava booster *+ Thiamethoxam 25G	7.10	3.37×10^{7}	13.31	1.49×10^{6}	15.93	8.84×10^{5}
T9	Control	14.59	1.21×10^{4}	14.89	1.31×10^{6}	12.54	2.40×10^{6}

*Proprietary product of TNAU, Coimbatore

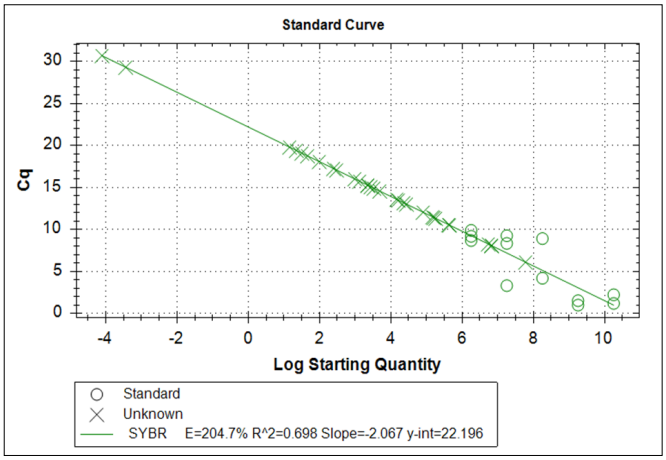

Fig. 1. Standard curve for pre -treatment sample in qPCR.

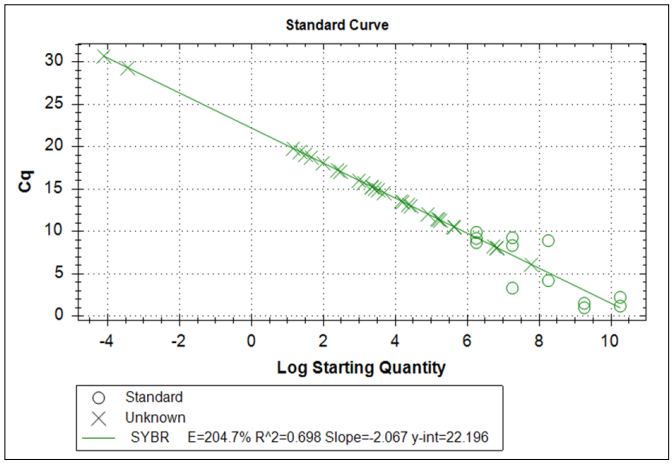

Fig. 2. Standard curve for treated sample in qPCR.

In contrast, control plants (T9) showed a steady increase in virus copy number, rising from 1.21×10^{4} to 1.31×10^{6} and then to 2.40×10^{6} at 0, 7 and 14 DAS, respectively. After one-week, viral concentrations rebounded in some treatments that had initially shown reductions. Of all the treatments, Treatment 3 (fish oil rosin soap) showed the most significant reduction in viral load, followed by Treatment 1 (cassava booster). A few samples were subjected to agarose gel electrophoresis to check for non-specific bands. Agarose gel electrophoresis is a commonly used method to evaluate the success of PCR reactions. It is one of the most reliable techniques for verifying the specificity of amplification, providing additional confirmation of qPCR results (Fig. 3).

Discussion

In this investigation, the effects of nutrient application and other treatments on cassava crops for managing CMD were evaluated using qPCR. The qPCR results revealed a decrease in viral copy numbers at 7 and 14 DAS, indicating that both fish oil rosin soap and cassava booster effectively reduced the virus concentration. Viral load assessment was performed using qPCR. A previous study reported that the relative concentration of viral DNA in symptomatic tissues was higher than in leaves showing recovery (25).

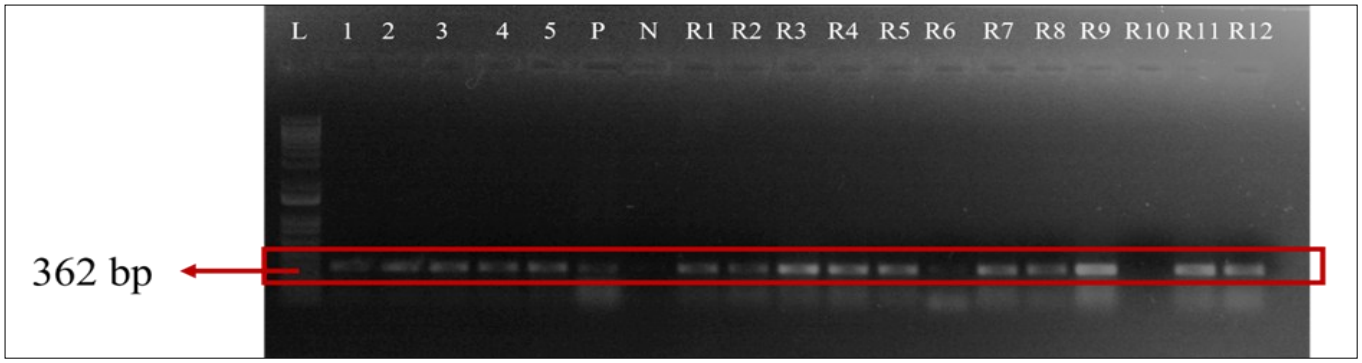

Fig. 3. Agarose gel electrophoresis of pre-treatment qPCR sample of SLCMV.

L- Molecular marker 4-Std 4 R1- R1T1 R5-R2T2 R9-R3T3
1-Std 1 5-Std 5 R2-R2T1 R6-R3T2 R10-R1T4
2-Std 2 P- P.C R3-R3T1 R7-R1T3 R11-R2T4
3-Std 3 N- N.C R4-R1T2 R8-R2T3 R12-R3T4

The results were reliable and reproducible, with low standard deviations in the Cq values and high correlation coefficients for the standard curves. The qPCR values were exported to CFX Maestro software (Version 2.3) to verify the standard curves of the optimized qPCR systems and to quantify target concentrations in the samples. The means and standard deviations of Ct values for triplicate samples were calculated. The Ct value for cassava booster (T1) steadily increased from 11.77 to 13.79 from 0 DAS to 14 DAS. For T3, the Ct value steadily increased from 13.16 to 20.93 during the same period of observation. The cycle threshold (Ct) values obtained from qPCR indicated that samples with higher Ct values had lower concentrations of the virus (26). Standard deviations between samples were evaluated using the log of the copy numbers. The copy numbers of DNA-A and DNA-B genome components were determined by interpolation from gene-specific standard curves.

Previous research quantified virus concentrations in the susceptible cassava cultivar TME 117 and the moderately resistant cultivar TMS 30572, which were inoculated with the EACMV UG (Ca 055 isolate) and the ACMV (DRC6 isolate) using a biolistic method. The virus concentration in symptomatic leaf tissues was significantly higher than in non-symptomatic leaves and this difference correlated with the severity of disease symptoms. The study found that EACMV-UG titers were higher than those of ACMV (27). Further, the quantitative evaluation revealed that both cultivars exhibited similar virus distributions. Both TME 117 and TMS 30572 had low levels of EACMV-UG in symptomatic tissues, suggesting that the presence of ACMV may suppress the accumulation of EACMV-UG. The qPCR method provides a rapid and reliable diagnostic tool for quantifying and differentiating between ACMV and EACMV-UG, which is essential for effective virus management in cassava cultivation (27).

An earlier study evaluated the ovicidal effects of different botanical treatments, including fish oil rosin soap and organic salts, on whitefly eggs using a direct spray method (28). The study found that fish oil rosin soap, though not as effective as cassava extract, significantly reduced egg hatchability and increased egg mortality. A similar result was obtained in the present study.

Another study discussed CMD management and its impact on cassava yield using a combination of nutrient and biocontrol agents (29). Foliar application of this combined mixture every 21 days from one to five months after planting significantly reduced CMD incidence by 65% compared to untreated control plants. Similarly, the micronutrient formulation cassava booster used in this study effectively reduced viral concentration. The foliar application of the micronutrient also increased the defense metabolites, particularly octadecatrienoic acid and trilinolein content.

A study on *Tomato Yellow leaf curl virus* (TYLCV) titer with regard to symptom severity using RT-PCR revealed a positive correlation between the viral titer and symptom severity. Samples collected according to the AVRDC severity scale (0 to 3) were subjected to qPCR. The highest viral accumulation was observed in plants classified as severity scale 3, with titer levels reaching up to 2.88×10^9 copies, while scale 0 samples had significantly lower viral copies (approximately 564 copies). Conventional PCR failed to detect the virus in asymptomatic plants (scale 0), but qPCR identified low levels of viral DNA, indicating that these plants could still harbor the virus. The cycle threshold (Ct) values obtained from qPCR showed that samples with higher disease severity (scales 1-3) had lower Ct values, reflecting higher viral loads (26).

Similarly, a study evaluating five bacterial endophyte treatments (*Bacillus velezensis* VB7, *Bacillus licheniformis* Soya 1, *Bacillus tequilensis* NBL9, *Bacillus sonorensis* KMR3 and *Myroides odorotimimus* YEBRT3) using qPCR showed a steady increase in *Groundnut Bud Necrosis Virus* (GBNV) copy numbers in all treatments over time, peaking at 96 hours. The virus count was highest in the inoculated control (1.2×10^8 copies). At the same time, bioagent-treated plants showed reduced virus levels, with *B. licheniformis* Soya 1 showing 2.4×10^7 copies, *B. velezensis* VB7 with 3.5×10^6 copies and *B. tequilensis* NBL9 with 3.6×10^6 copies, indicating that the bioagents successfully suppressed viral replication (30).

Conclusion

Cassava is a versatile crop that will remain crucial for both food and industrial purposes in the future. However, its potential has been significantly threatened by viral diseases. This study explored integrated nutrient and disease management treatments to mitigate the impact of CMD. The results from the different treatments indicated that fish oil rosin soap, a naturally derived product, initially reduced the viral concentration. At the same time, the cassava booster gradually decreased the virus titer in the cassava plants, potentially reducing disease severity. The precise quantification of viral load using qPCR provides a valuable diagnostic tool for monitoring infection levels in cassava plants. This integrated approach not only helps to reduce CMD severity but also offers a more sustainable framework for cassava crop protection, ensuring food security and improved yields. It lays the groundwork for further research into CMD management using environmentally friendly practices, such as combinations of nutrients, naturally derived products like fish oil rosin soap, azadirachtin and sticky traps, all of which may contribute to enhanced cassava production.

Authors' contributions

BR carried out all the treatment works and qPCR analysis and drafted the manuscript. LTKS contributed to language editing and manuscript correction. MT contributed in the interpretation of the results. HS and VM contributed to the manuscript correction. SPA contributed to revising the treatments.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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