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Research Article

Validation and Standardization of Revers Transcription-Polymerase Chain Reaction (RT-PCR) Techniques for *Cassava Brown Streak Virus* (CBSV) Detection

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Abstract

Cassava (Manihot esculenta) is a vital staple food crop in tropical regions, providing a significant source of nutrition and income for millions of people, especially in sub-Saharan Africa. However, Cassava brown streak disease (CBSD) poses a significant threat to its production, causing substantial yield losses and rendering cassava roots economically unusable. This study aims to validate the RT-PCR technique for the accurate and timely detection of Cassava brown streak virus (CBSV), the causative agent of CBSD. To achieve this, cassava leaf samples showing typical CBSD symptoms were collected from Mozambique, Tanzania, and Uganda. These samples were then subjected to RNA extraction using a modified Cetyl trimethylammonium bromide (CTAB) protocol at the Natural Resource Institute (NRI). The RNA was extracted and used as a template for reverse transcription-polymerase chain reaction (RT-PCR) with specific primers targeting CBSV. The results show successful amplification of CBSV partial coat protein sequences in all tested samples using CBSV 10 and 11 primers, confirming the presence of CBSV. Additionally, CBSV 9 and 11 primers successfully amplify full-length coat protein sequences from two samples, providing further validation of CBSV presence. These findings highlight the importance of RT-PCR as a sensitive and specific technique for detecting CBSV, providing a valuable tool for disease surveillance and management in cassava production. The study reinforces the significance of addressing CBSD to ensure food security and economic stability in regions that rely on cassava cultivation. Further research and implementation of RT-PCR-based diagnostic methods could help to reduce the socio-economic impact of CBSD and promote sustainable cassava production.

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Introduction

Cassava (*Manihot esculenta*) is a shrubby, dicotyledonous, and perennial plant that belongs to the genus Manihot of the family Euphorbiacea (Saggaf *et al*., 2019). It is a starchy root crop that grows to a height of 2-3 metres and requires 8-11 months in the ground before producing a usable yield (Howeler, 2002). Cassava is a staple food crop in tropical countries, with over 800 million people worldwide consuming it either directly or indirectly daily (Elegba *et al*., 2020; Patil and Fauquet, 2009). Cassava is the second most important food in Africa in terms of calories consumed, after maize. It plays two major roles: providing food security for many households and serving as raw material for agro-industrial development. In 2017, over 291 million tons of cassava were produced globally, with Africa accounting for more than 60% of the total production (IITA, 2021; Olukunle and Olukunle, 2007; Thro *et al*., 1996). Cassava is primarily a subsistence crop grown for food by small-scale farmers in sub-Saharan Africa (SSA) who sell the surplus (IITA, 2021). According to Pearce (2007), in some parts of Africa, cassava is the only crop preventing millions of people from starving. For instance, in Mozambique, MOAF (1994) reported that half of the population depends on cassava tubers for consumption.

However, cassava propagation is vegetative, through stem cuttings, and its extended stay on the ground before harvest makes the plant more susceptible to several diseases, including at least 16 different viruses (Calvert and Thresh, 2002; Bellotti, 2002). Some viruses are transmitted from crop to crop by whiteflies, including *Bemisia tabaci* and *Bemisia afer*. There are approximately 11 whitefly species known to feed on cassava (Bellotti *et al*., 1999; Saggaf *et al*., 2019). Although cassava is important to many households, particularly in Africa, its production and utilization have decreased due to various diseases caused by viruses, bacteria, fungi, and nematodes (Hillocks and Wydra, 2002). Cassava brown streak disease (CBSD), cassava mosaic disease (CMD), and cassava bacterial blight (CBB) are among the major diseases affecting cassava. Cassava Mosaic

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Disease (CMD) is responsible for a significant portion of cassava root loss in sub-Saharan Africa, resulting in yield losses of approximately US $1 billion annually (Elegba *et al*., 2020; Legg *et al*., 2011).

Cassava brown streak disease (CBSD) is a significant threat to cassava production in Africa, rendering the starch storage roots economically unusable (Alicai *et al*., 2016; Hillocks *et al*., 1999). CBSD was first reported in what is now Tanzania by Storey in 1936. The disease was named 'Brown Streak' due to the presence of brown, elongated necrotic lesions that can be found on the green stem tissue of infected plants (Storey 1936; Hillocks *et al*., 1996; Patil *et al*., 2015). Cassava Brown Streak Disease (CBSD) has two synergistic effects on the plant: one on root quality and the other on the total yield of the crop, which affects the marketability of cassava (Alicai et al., 2016; Winter *et al*., 2010). According to research by Alicai *et al*. (2016) and Hillocks *et al*. (2001), CBSD can reduce cassava root weight by up to 70% in the most susceptible cultivars, making it economically unviable. The symptoms of infected plants, as noted by Hillocks *et al*. (1999), vary depending on crop age, cultivar, and environmental conditions. Cassava yield losses due to CBSD were estimated to be over US $100 million per year (CGIAR, 2003). The causative agent, Cassava brown streak virus (CBSV), was not classified until 2001 when it was identified as a member of the genus Ipomovirus in the family Potyviridae and confirmed as the cause of the disease (Monger *et al*., 2001a).

RT-PCR has been shown to be a sensitive and highly specific technique for detecting viruses in host organisms that are difficult to detect by serology or electron microscopy (Seal and Coates, 1998; Jalali *et al*., 2017). The aim of this study was to validate the RT-PCR technique of Monger *et al*. (2001b) for CBSV at the Natural Resource Institute.

Significance of Study

The study's significance lies in addressing a critical issue affecting cassava production, particularly in sub-Saharan Africa. Cassava serves as a staple food crop for millions of people in the region. Cassava brown streak disease (CBSD) poses a significant threat to food security and economic stability, leading to substantial yield losses and rendering cassava roots economically unusable. This study contributes to the development of reliable diagnostic tools essential for disease management and control by validating the RT-PCR technique for detecting Cassava brown streak virus (CBSV). Successful validation of this technique can facilitate early detection and timely intervention,

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ultimately safeguarding cassava production and ensuring food security for vulnerable communities relying on this crop.

Statement of Problem

Cassava brown streak disease (CBSD) is a significant challenge to cassava production in Africa, resulting in substantial yield losses and economic impact. The disease affects both root quality and total crop yield, rendering cassava cultivation economically unviable for many small-scale farmers. Despite the devastating consequences of CBSD, detecting Cassava brown streak virus (CBSV) accurately and promptly remains a challenge. Traditional diagnostic methods are criticised for their lack of sensitivity and specificity, which hinders effective disease management strategies. The study addresses the need for a reliable and efficient diagnostic technique for CBSV to enable early detection and intervention, thereby mitigating the impact of CBSD on cassava production and food security.

Justification

The study is justified by the crucial role cassava plays as a staple food crop for millions of people in sub-Saharan Africa. The prevalence of CBSD poses a threat to livelihoods and food security in the region. Therefore, it is essential to have a reliable method for detecting CBSV to implement targeted disease control measures and prevent further spread of the virus (Maruthi *et al*., 2014). The RT-PCR technique, proposed by Monger et al. (2001b), is a promising solution due to its sensitivity and specificity in detecting viral pathogens. Validating this technique at the Natural Resource Institute will enhance its credibility and applicability, providing researchers and stakeholders with a valuable tool for disease surveillance and management. The validation of RT-PCR for CBSV detection has the potential to mitigate the socio-economic impact of CBSD and contribute to sustainable cassava production in the region.

Materials and Methods

Sample collection

Cassava plants showing symptoms of CBSD were originally from Mozambique, Tanzania and Uganda but subsequently maintained in the NRI quarantine glasshouse. Fresh leaves samples showing typical disease symptoms were collected from the growing cassava plants at the NRI quarantine glasshouse, and placed into labelled plastic bags for viral-RNA extraction.

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Extraction of RNA from cassava leaves

A modified CTAB protocol for total nucleic acid extraction by Maruthi *et al.*, (2002), adapted from Lodhi *et al*. (1994) was used. The 2% CTAB extraction buffer was first preheated in a water bath for 10 minutes at 60^{o}C. About 100mg (0.1g) of cassava leaf from each infected sample was ground thoroughly using a roller mixed with 1 ml (1000 µl) of extraction buffer in a thick gauged plastic bag. Approximately 750 µl of each sample was heated at 60^{o}C for 30 min, then mixed with an equal volume (750 µl) of phenol: chloroform: isoamylalcohol (25:24:1) by vortexing and then centrifuged for 10 minutes at 13000 rpm. Ice cold (-20^{o}C) isopropanol of about 0.6 volumes (300 µl) was added precipitate nucleic acids and incubated at -20^{0}C for at least 1 h, then centrifuged at 13000 rpm at 4^{o}C for 10 min. The pellet washed with ethanol was suspended in 100 µl 1x TE buffer and stored at -20^{o}C for analysis.

Primers used in Polymerase Chain Reaction

The Primers used for detection of CBSV in cassava (Monger *et al.*, 2001b). CBSV9 (ATGCTGGGGTACAGACAAG), CBSV11(CCACATTATTATCGTCACCAGG) CBSV10 (ATCAGAATAGTGTGACTGCTGG), CBSV11(CCACATTATTATCGTCACCAGG). A two-step process; reverse transcription and polymerase chain reaction were performed to amplify the virus genome segments.

Reverse Transcription (RT)

Samples were heated at 65 ^{o}C for 5 min. and quickly placed on ice for 2 min. Total reaction volume 10 µl (Superscript RT, dNTPs (2.5 µM), Oligo dT (30 µM), Samples) were incubated in water bath at 42^{o}C for 50 min. Reaction in the sample was inactivated by heating at 75^{o}C for 15 min. The cDNA so generated was used as template for amplification by PCR,

Cycling condition of PCR amplification

PCR reaction mixture (distilled water, 10 X buffer, $MgCl_2$ (25mM), dNTPs (2.5 mM), CBSV 10 primer (20µM), CBSV 11 primer (20µM), Red hot polymerase (5U) and cDNA) was transferred into 0.5 µl PCR tubes. The PCR tubes were then transferred to a PCR machine (thermal cycler) programmed as 1 cycle at 94^{o}C/2 min, 35cycles at 94^{o}C/45 s for denaturation, 55^{o}C/45 s for annealing and 72^{o}/ 1 min for DNA synthesis and 1cycle at 72^{o}C/10 min for final extension the reaction was kept hold at 20^{o}C. The PCR products and 5X orange G dye were run using 1.2% gel electrophoresis in 0.5X TBE buffer. It was then stained in ethidium bromide (0.5 µl /ml) and the bands were visualised on a UV (302 nm) transilluminator and photographed.

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Results and Discussion

RNA extraction from cassava leaves

Total RNA was extracted from cassava leaf material showing typical symptoms of CBSD. CBSD samples were originally obtained from Dar-es-Salam and Naliendele in Tanzania, Kyabanole in Uganda and Nampula in Mozambique. A CTAB nucleic acid extraction protocol, modified from that used by Lodhi *et al.* (1994) was applied for the extraction of total RNAs. Readings obtained using the spectrophotometer indicated that the method provided RNA of sufficient quality and quantity for successful RT-PCR tests.

One-step RT-PCR using CBSV 10 and CBSV 11 primers

Viral RNA amplification for CBSV detection was carried out using CBSV 10 and 11 primers originally developed by Monger *et al.* (2001a). The technique involved the production of cDNA first and followed by amplification of cDNA in a single reaction. PCR products of expected sizes (~230 bp, fig. 1) were obtained using these primers which indicated that the extracted RNA from cassava leaves were of sufficient quality for RT-PCR tests, as also reported by Lodhi *et al.* (1994). Positive results in this PCR test also indicated the presence of CBSV in cassava samples obtained from the above-mentioned locations.

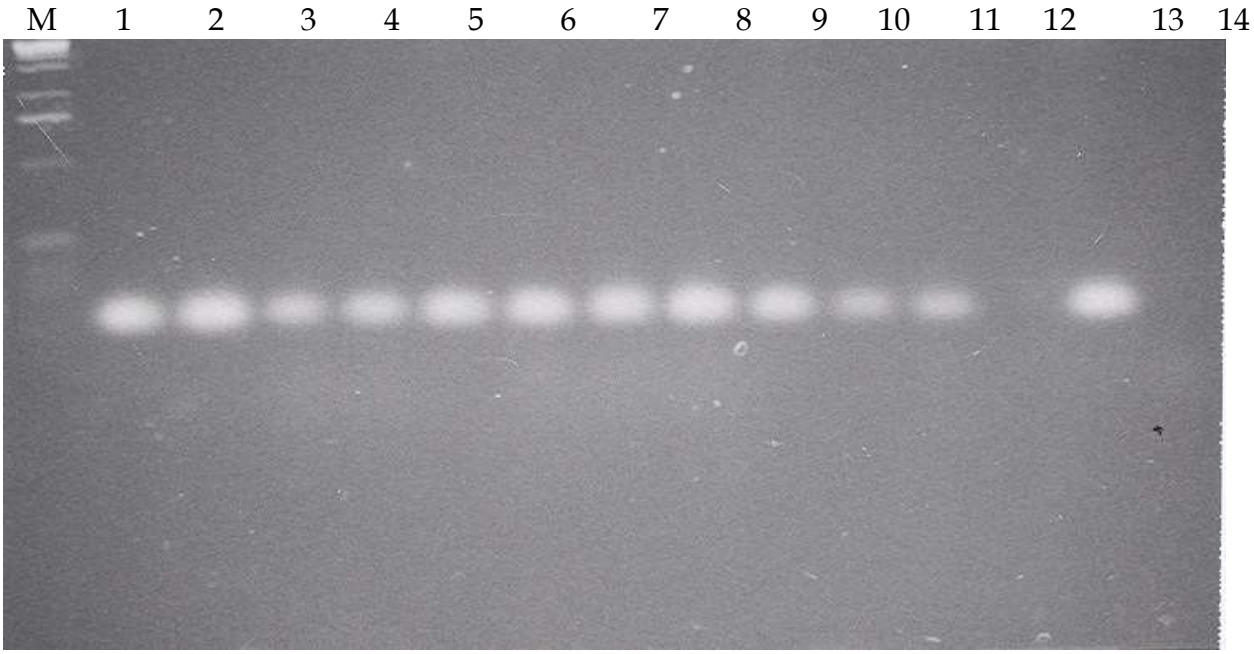

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Figure 1. Gel photograph of PCR products generated using CBSV 10 and 11 set of primers indicating the successful detection of CBSV in different cassava isolates (tracks 1-11). M = marker (1 kb Gibco-BRL). Lane 1-2 Nampula M1, 3-4 Nampula M2, Lane 5-6 Dar-es-Salaam (whitefly transmitted), 7-8 Naliendele, Lane 9-10 Kyabanole and Lane 11-12 Dar-es-Salaam. Lane 13 is a positive control (previous RNA extractions) and lane 14 a negative control (water added instead of RNA template).

Two-step RT-PCR using CBSV 9 and CBSV 11 primers

In the first step, cDNA was generated from reverse transcription (RT) with an OligodT primer in an attempt to amplify full-length CBSV coat protein. In the second step, PCR was performed using virus-specific primers (CBSV 9 and 11) which successfully amplified products from two samples namely Nampula M2 from Mozambique (fig. 2, lanes 3-4) and from Naliendele from Tanzania (fig. 2, lanes 7-8). Serial dilution of samples (10-, 100- and 1000-fold) produced no visible band (fig. 2). The inability to amplify full-length CBSV CP gene sequences from the other isolates was consistent with Monger *et al.,* (2001a) where inconsistent amplifications were obtained.

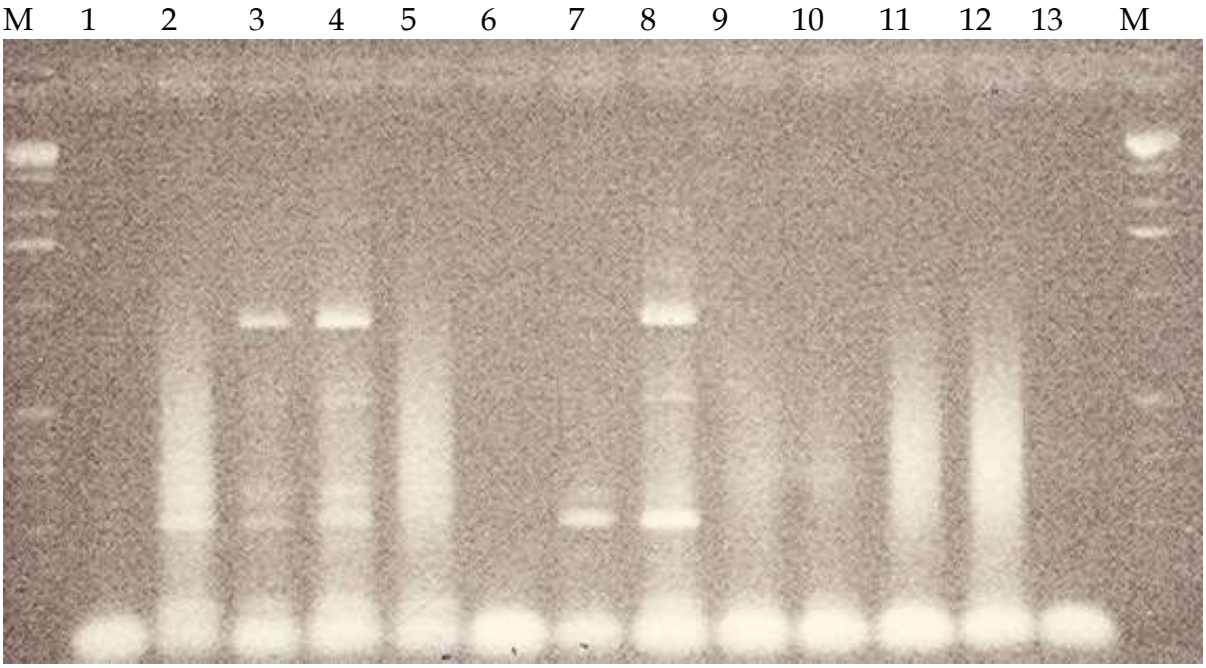

Figure 2. Gel photograph of PCR products generated using CBSV 9 and 11 primers showing the successful amplification of CBSV full-length coat protein from isolates Nampula M2 from Mozambique and Naliendele from Tanzania. M = size marker (1 kb

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Gibco-BRL). Lanes 1-2 Nampula M1, 3-4 Nampula M2, 5-6 Dar-es-Salaam (whitefly transmitted), 7-8 Naliendele, 9-10 Kyabanole and 11-12 Dar-es-salaam (old leaf), while lane 13 represented negative no template control and M = 1 kb size marker.

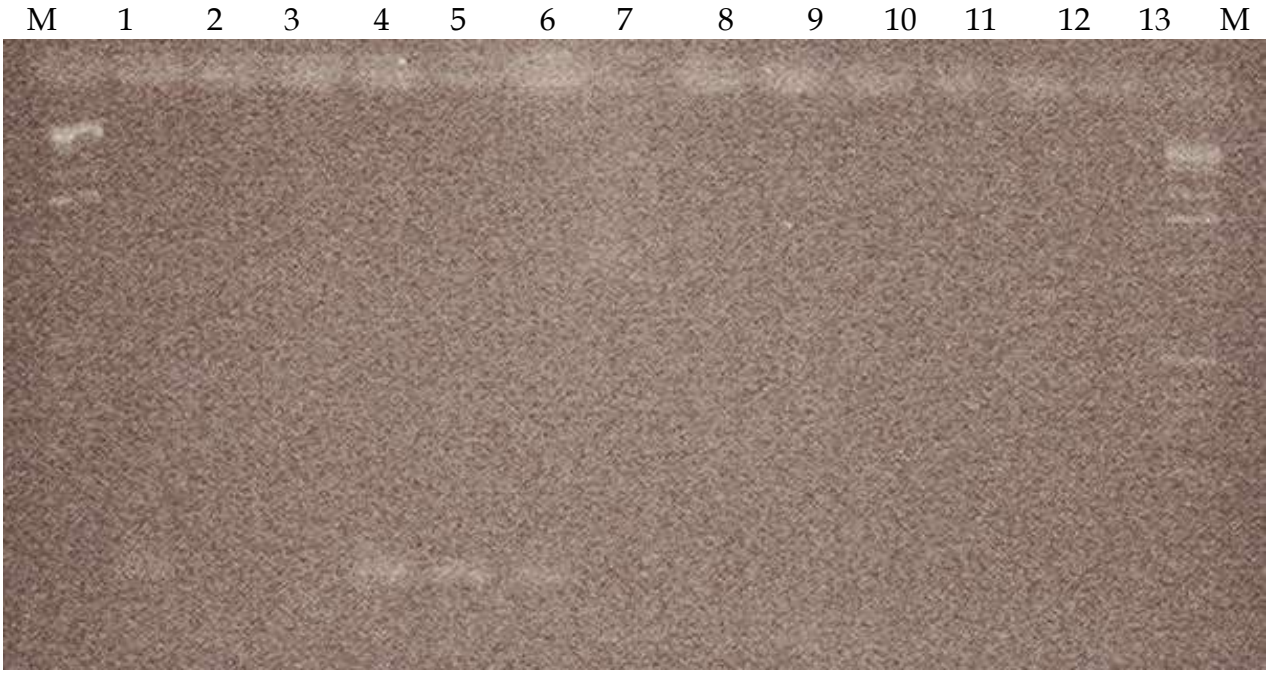

Figure 3. Gel photograph of PCR products generated using CBSV 9 and 11 primers showing the failed amplification of CBSV full-length CP upon serial dilution of DNA samples. M = size marker (1 kb Gibco-BRL). Lanes 1-2 Nampula M1, 3-4 Nampula M2, 5-6 Dar-es-Salaam (whitefly transmitted), 7-8 Naliendele, 9-10 Kyabanole and 11-12 Dar-es-salaam (old leaf), while lane 13 represented negative control and M = 1 kb size marker.

Conclusion

Cassava sticks suspected of having CBSD were originally collected from three African countries (Mozambique, Tanzania and Uganda) and transferred to the NRI quarantine glasshouse. Leaves showing CBSD symptoms from the resulting cassava plants were collected and total nucleic acids extracted using a CTAB extraction method which generated templates of sufficient quality and quantity for RT-PCR diagnostic tests. RT-PCR primers CBSV 10 and 11 primer set amplified partial CP sequences from all the tested samples while the primer set CBSV 9 and 11 amplified full-length CP from only

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two of the samples. These results confirmed earlier reports (Monger *et al*., 2001a) that CBSV 10 and 11 were the most reliable RT-PCR primers for CBSV diagnosis, and the RT-PCR tests indicated that all the three cultivars were CBSV positive.

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