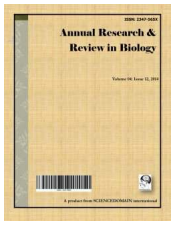

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Rapid and Efficient Isolation of High Quality DNA from Cassava (*Manihot esculenta* Crantz) Suitable for PCR Based Downstream Applications

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ENN and NOA designed the study. Author GO carried out the laboratory experiments, performed statistical analysis and wrote the first draft of the manuscript. Authors ENN and NOA supervised the laboratory experiments and offered technical advice. All authors read, reviewed and approved the final manuscript.

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ABSTRACT

The extraction of high-quality DNA from cassava leaves suitable for various molecular techniques is a challenge due to the presence of polysaccharides, proteins and polyphenols that interfere with the isolation procedures and downstream applications. This article describes a rapid and efficient procedure for isolating high yield and quality DNA from cassava leaves of six different cultivars (Kibandameno, Seveu, Mkombozi, TMS60444, TME14 and TME419). Improvement on the quantity and quality of the extracted DNA was achieved through modification of cetyl trimethylammonium bromide (CTAB) DNA extraction procedure. The modifications included addition of 20% sodium dodecyl sulfate (SDS) and 4% polyvinylpyrrolidone (PVP), use of increased concentration of ethylenediaminetetraaceticacid (EDTA) and exclusion of liquid nitrogen. The quantity and quality of extracted DNA was assessed using a spectrophotometer and agarose gel electrophoresis. The modified method in this study yielded an average amount of 2400.5 - 2919.8 ng/µl per 100 mg of

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leaf materials with UV absorbance ratios A260/280 of 1.81 - 1.85. Agarose gel electrophoresis (1%) illustrated intact, sharp and clear bands without degradation. The isolated DNA with this protocol served as a robust template for PCR based downstream applications of simple sequence repeats (SSR) and virus detection.
The results presented in this study demonstrate the suitability of CTAB-SDS method in yielding high quality DNA from cassava leaves suitable for downstream molecular biology techniques.

Keywords: Manihot esculenta Crantz; cetyl trimethylammonium bromide (CTAB); DNA extraction; simple sequence repeats; virus detection.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) provides a staple food and income generation for over 800 million people worldwide. The importance of cassava as a food and industrial crop relies on its roots since they accumulate starch, and is the second source of starch globally, after maize [1]. There has been an increase in the demand for cassava, particularly due to its use as animal feed and as a source of raw material for production of bioethanol and industrial starch. Crop improvement facilitated by modern biotechnology has largely been acknowledged as a key strategy for achieving food security and sustainable agriculture [2]. The basic step for many techniques of biotechnology, such as molecular markers, molecular diagnostics and genetic engineering, aimed at genetic improvement of cassava, is the ability to isolate high quality genomic deoxyribonucleic acid (DNA) suitable for diverse polymerase chain reaction (PCR) applications.

The ability to extract high-quality DNA from cassava is problematic due to the high presence of polysaccharides, polyphenolics and other compounds [3]. Phenolic compounds are readily oxidized to form covalently linked quinones, which bind irreversibly to nucleic acids and proteins forming a gelatinous complex that gives the DNA a brown color [3]; the polysaccharides tend to co-precipitate with the DNA in the presence of alcohols, remaining as contaminants in the final extract, known to inhibit polymerase activity. This drastically reduces both the yield and quality of the resultant DNA. Both contaminants also prevent the use of DNA for molecular biology purposes, such as PCR, restriction digestions, or sequencing by inhibiting the action of polymerases or endonucleases [4,5].

Various methodologies of DNA isolation from plant species rich in polyphenols or polysaccharides have been reported [6,7,8,9, 10]. Although these methodologies have been reported for DNA isolation from specific plant tissues, they necessarily cannot be applied for other plant tissues [10] and thus ultimately raise the need to explore different DNA extraction protocols for certain plant materials. The cetyl trimethylammonium bromide (CTAB) method and its modifications have been used to obtain good quality total DNA for PCR based downstream applications. Commercial extraction kits such as DNeasy Plant Mini kits (Qiagen, GmbH, Germany) are available, but they may not be suitable for plants rich in polyphenolic and polysaccharide compounds and they can be relatively expensive [11,12]. Therefore, there is need to develop CTAB-based protocols to suit DNA extraction from specific plant species including cassava [13,14,15]. The reliable quality of DNA is a basic requirement for subsequent PCR based applications. Polymerase chain reaction has found wide applications in genomic studies. For reproducible PCR results, conversely, the quantity and quality of DNA play an important role.

In the present study, we describe a simple, rapid, reliable and efficient CTAB based method for the extraction of high quality total DNA of different cassava cultivars and associated geminiviruses. The isolated high quality genomic DNA is amenable to simple sequence repeats (SSR) amplification and detection of *East African cassava mosaic virus* (EACMV) associated with cassava. The quantity and quality of DNA extracted by this method was compared with a commercial kit and CTAB methods.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh, young leaves of six cassava cultivars namely Kibandameno, TMS60444, TME14, TME419, Mkombozi and Seveu were collected from the glasshouse at the School of Biological Sciences, University of Nairobi. For PCR based

virus detection, symptomatic leaves of cassava plants of mentioned cultivars naturally infected with EACMV were collected. A minimum of five replicates were taken from each cultivar.

2.2 Reagents and Chemicals

All the reagents and chemicals used in the present study are mentioned in Table 1.

2.3 Modified DNA Extraction Protocol (CTAB-SDS Based)

1. CTAB extraction buffer (100 mM Tris-HCl (pH 8), 1.4 M NaCl, 0.2 M EDTA (pH 8), 4% (w/v) PVP and 2% (w/v) CTAB) was pre-heated in a water bath at 60℃ for 20 minutes.
2. Leaf samples weighing 100 mg were collected in a 1.5 ml centrifuge microtube on ice, and 500 µl of CTAB buffer plus 150 µl of 20% (w/v) SDS were added and ground with a plastic pestle to homogenize the tissue.
3. The homogenate was incubated in a 55℃ water-bath for 20 minutes with inversions of the microtubes 3 – 5 times after every five minutes.
4. The homogenate was centrifuged at 13,800 rpm at room temperature for 5 minutes and the supernatant carefully transferred to a new microtube using a 200 µl micropipette.
5. An equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed by inversion for 5 minutes.
6. The sample was centrifuged at 13,800 rpm at room temperature for 5 minutes and the supernatant carefully transferred to a new microtube.
7. Again an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed by inversion for 5 minutes.
8. The sample was centrifuged at 13,800 rpm for 5 minutes and the upper phase transferred to new microtubes followed by addition of 50 µl of 7.4 M ammonium acetate and 2 volumes of ice cold absolute ethanol.
9. The tubes were incubated at -20℃ for 20 minutes to precipitate nucleic acids from the solution.
10. The microtubes were centrifuged at 10,000 rpm for 5 minutes, supernatant discarded and the pellet collected.
11. The pellet was washed with 500 µl of a wash solution (75% ethanol and 15 mM ammonium acetate). The microtubes with the pellet were centrifuged at 10,000 rpm for 5 minutes after every wash, and the supernatant discarded. The washing step was repeated twice.
12. The pellet was dried by inverting the microtube onto the paper towel for 10 minutes until the ethanol evaporated completely.
13. To dissolve the DNA, 70 µl of TE buffer was added followed by 3 µl of 10 mg/ml ribonuclease A to digest RNA.
14. The DNA was incubated at 37℃ for 30 minutes in a water bath and stored at -20℃ for further use.

2.4 Other DNA Extraction Protocols

The method described by Devi et al. [16] and commercial kit DNeasy™ plant mini kit (Qiagen, GmbH, Germany) were used as controls for comparison with the proposed extraction method.

2.4.1 Improved CTAB method described by Devi et al. [16]

Extraction buffer (100 mM Tris–HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 8), 2% (w/v) CTAB) was pre-heated in a water bath at 60℃ for 15 minutes. One hundred grams of cassava leaf tissue was submerged in 5 ml of absolute ethanol for 5 minutes and ethanol allowed to evaporate. In the presence of 1% PVP and pre-warmed CTAB extraction buffer, the tissue was ground using a pre-chilled mortar and pestle at room temperature. The homogenate was transferred into 1.5 ml centrifuge microtubes and incubated in water bath at 60℃ for 1 hour. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4℃ and the supernatant transferred to new microtubes. Equal volume of chloroform:isoamyl alcohol (24:1) was added and samples were mixed by inversion for 15 minutes. The microtubes were centrifuged at 10,000 rpm for 10 minutes at 4℃. The step was repeated and supernatant transferred into new centrifuge microtubes. Two volumes of chilled isopropanol was added and incubated at −20℃ for 30 minutes to precipitate the DNA. The microtubes were centrifuged at 10,000 rpm for 10 minutes to pellet down the DNA. The pellet was washed twice with 70% ethanol and air dried at room temperature. The pellet was dissolved in 70 µl of TE buffer.

Table 1. Chemicals and reagents used in DNA extraction

Serial number	Components/Chemicals	Concentrations
1	Cetyltrimethylammonium bromide (CTAB)	2% (w/v)
2	Sodium chloride (NaCl)	1.4 M
3	Ethylenediaminetetraacetic acid (EDTA) (pH 8.0)	0.2 M
4	Tris-HCl (pH 8)	100 mM
5	Polyvinylpyrrolidone (PVP)	4% (w/v)
6	Sodium dodecyl sulfate (SDS)	20%
7	Chloroform-isoamyl alcohol	24:1
8	Ammonium acetate	7.4 M
9	Absolute ethanol	100%
10	Wash solution:	
	(i) Ethanol	75%
	(ii) Ammonium acetate	15 mM
11	TE buffer (pH 8):	
	(i) Tris-HCl	10 mM
	(ii) EDTA	1 mM
12	Tris/Acetate/EDTA	1X
13	Agarose (molecular grade)	1% (w/v)

2.4.2 Column and kit based DNA isolation method

We have selected a standard DNA isolation kit commercially named as DNeasy™ plant mini kit (Qiagen, GmbH, Germany) which ensures good quality DNA recovery from plants. All the steps were followed as per manufacturer's instructions. The eluted DNA was finally stored at -20℃ for further use.

2.5 Quantification and Visualization of DNA

DNA samples were subjected to agarose gel electrophoresis in 1X TAE (Tris Acetate- EDTA) buffer for 65 minutes at 60 Volts. To check the DNA quality, 5 µl of each sample was loaded on 1% (w/v) agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized under UV transilluminator. The gels were photographed using Easy Doc plus gel documentation system. The DNA from different cassava cultivar samples were quantified using a spectrophotometer (UV–Visible Elico spectrophotometer) at A_{260}, while purity of the DNA was checked through A_{260}/A_{280}.

2.6 Simple Sequence Repeats (SSR) Analysis

To assess the quality of the cassava DNA and its suitability for PCR, the microsatellite sequence SRY9 was amplified [17]. PCR amplifications of genomic DNA samples extracted from different cassava cultivars using three different methods were carried out using SSR primer (SRY9). The SRY9 primer (forward; 5'ACAATTCATCATGAGTCATCAAC3' and reverse; 5'CCGTTATTGTTCCTGGTCCT3') was developed by Mba et al. [17] and the primer used in this study was synthesized by Inqaba Biotec (South Africa).

The PCR amplifications were carried out in a total volume of 20 µl containing 20 ng of genomic DNA, 4 µl of 5X PCR buffer containing 15 mM $MgCl_2$ and 0.2 mM dNTPs, 1 unit Taq polymerase (Bioline, USA) and 10 µM of each SSR primer. PCR amplifications were carried out in a MJ Mini™ personal Thermal Cycler (Bio-Rad, Singapore) using the following thermal cycling conditions: initial DNA denaturation at 95℃ for 5 minutes, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 54℃ for 30 seconds and extension at 72℃ for 1 minute with a final extension at 72℃ for 7 minutes. The samples were then maintained at 10℃.

2.7 Geminivirus DNA Detection

East African cassava mosaic virus (EACMV) was detected by PCR using specific primers of EACMV: EAB555F (forward primer) 5'-TACATCGGCCTTTGAGTCGCATGG-3' and EAB555R (reverse primer) 5'-CTTATTAACGCCTATATAAACACC-3' [18]. The PCR amplifications were carried out in a total volume 20 µl containing 20 ng of genomic DNA, 4 µl of 5X PCR buffer containing 15 mM $MgCl_2$ and 0.2 mM dNTPs, 1 unit Taq polymerase (Bioline, USA) and 10 µM of each primer. PCR amplifications were carried out in a MJ Mini™ personal Thermal Cycler (Bio-Rad, Singapore)

using the following thermal cycling conditions: initial DNA denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 58℃ for 30 seconds and extension at 72℃ 40 seconds with a final extension at 72℃ for 7 minutes. The samples were then maintained at 10℃.

For both SSR analysis and EACMV detection, amplified products were electrophoresed in 1.5% (w/v) agarose gels in 1X TAE and stained with ethidium bromide (0.5 µg/ml). The gels were photographed under Easy Doc plus gel documentation system.

3. RESULTS AND DISCUSSION

The CTAB protocol is widely used for extraction of DNA from plant tissues. In the present study, the modification of CTAB protocol [16] facilitated the isolation of high-quality and quantity DNA from cassava, a plant rich in polysaccharides and polyphenols. This modified method (CTAB-SDS) was compared with two different DNA isolation protocols (CTAB and DNeasy plant mini kit) using leaf samples of 6 cassava cultivars. To ensure comparability between procedures, aliquots of the same leaf for all the cultivars were used for all protocols. In that way, plant material at the same developmental stage was used in all the procedures. The DNA extraction protocol (CTAB-SDS) described herein yielded high quantity and quality DNA without degradation from leaf tissues of different cassava cultivars (Table 2). The average yield of total DNA from 100 mg of leaf material using our method (CTAB-SDS) ranged from 2400.5 - 2919.8 ng/µl which was significantly higher than those obtained with commercial kit and CTAB method (Table 2). The A_{260}/A_{280} ratio was in the range of 1.81 - 1.85 (Table 2) which indicated the purity of the DNA obtained using our method and insignificant levels of proteins and polysaccharide contaminants.

In this study, DNA quality was assessed by agarose gel electrophoresis and clear, sharp and high molecular weight bands without degradation were obtained, indicating a high quality of isolated DNA samples (Fig. 1[I] and [II]). Total DNA isolated using CTAB method [16] and commercial kit did not produce distinct and intact bands (Fig. 1[I] and [II]) and there were presence of smeared bands indicating degradation. The results show that modified CTAB-SDS method gave intact DNA, while those of CTAB and commercial kit gave sheared DNA bands. The success of the CTAB-SDS extraction method in obtaining high-quality total DNA from all the tested cassava cultivars demonstrated the broad applicability of the method. Although, better yield was obtained using commercial kit method, the quality of the bands was not good and there were smears indicating degradation of DNA (Fig. 1[II]).

Table 2. Spectrophotometric results represent the yields and quality of DNA extracted from leaves of different cassava cultivars using three different extraction methods

DNA extraction method	Cassava cultivar	DNA concentration (ng/µl)*	A_{260}/A_{280}*
CTAB-SDS	TME419	2919.8 ± 2.04a	1.83 ± 0.01
	Kibandameno	2509.7 ± 21.78a	1.85±0.01
	TME14	2817.3 ± 11.36a	1.82±0.01
	Seveu	2400.5 ± 19.94a	1.83±0.01
	Mkombozi	2613.1 ± 7.69a	1.81±0.01
	TMS60444	2701.4 ± 8.36a	1.85±0.01
CTAB (Devi et al., 2013 [16])	TME419	578.4 ± 15.78c	2.33±0.06
	Kibandameno	433.6 ± 12.47c	1.45±0.08
	TME14	516.7 ± 13.42c	1.62±0.06
	Seveu	816.7 ± 5.26c	1.75±0.19
	Mkombozi	716.7 ± 6.24c	1.54±0.04
	TMS60444	713.7 ± 8.22c	1.64±0.03
DNeasy™ plant mini kit	TME419	1280.5 ± 10.74b	1.81±0.01
	Kibandameno	1534.3 ± 13.07b	1.83±0.01
	TME14	1827.2 ± 13.38b	1.81±0.01
	Seveu	1730.4 ± 5.77b	1.80±0.03
	Mkombozi	2100.7 ± 5.31b	1.83±0.02
	TMS60444	1998.7 ± 10.78b	1.80±0.01

**Mean data of 5 samples*

**Means followed by same letter(s) in a column indicate non-significant differences (p > 0.05)*

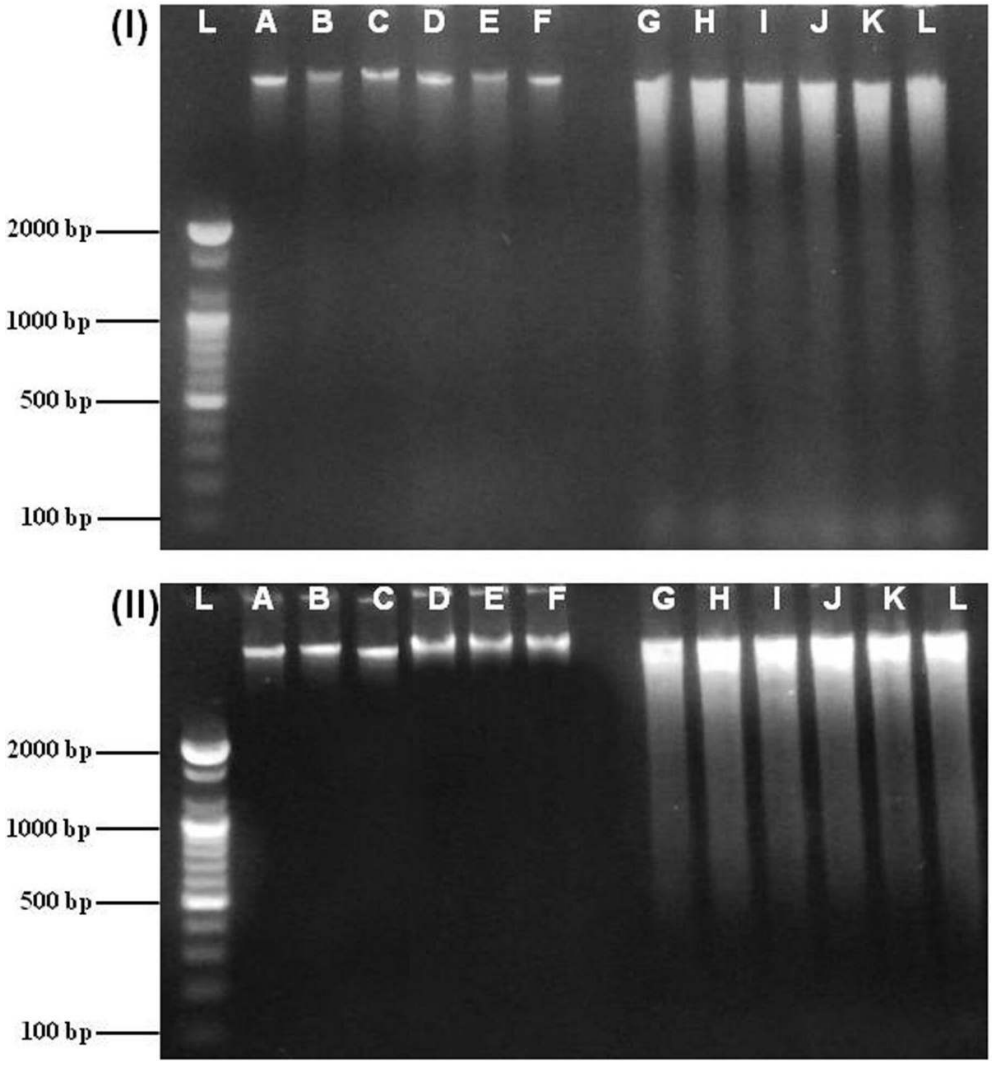

Fig. 1. Electrophoresis of total DNA extracted from leaves of different cassava cultivars using (I) CTAB-SDS (A - F) and CTAB (G - L) and (II) CTAB-SDS (A - F) and DNeasy™ plant mini kit (G - L) methods. Lane L - 100 bp molecular weight marker (Bioneer) and Lanes A – F represents DNA samples isolated from cultivars Kibandameno, Seveu, Mkombozi, TMS60444, TME14 and TME419, respectively. Lanes G – L represents DNA samples isolated from cultivars in same order as Lanes A-F

Majority of DNA extraction methods from plant leaf tissue are derived from the original CTAB based method, described by Doyle and Doyle [6]. In the present study, the proposed modifications to the CTAB method included the use of both 2% CTAB and 20% SDS in the same protocol, use of 4% Polyvinyl pyrrolidone, use of increased EDTA concentration (0.2 M) and exclusion of liquid nitrogen during tissue grinding. This method facilitated the isolation of large quantities of high-quality DNA from different leaves of cassava cultivars. The three main contaminants associated with plant DNA that can cause considerable difficulties while conducting PCR experiments are polyphenolic compounds, polysaccharides, proteins and RNA. Use of 20% SDS during grinding improved the amounts of DNA yield in the present study. Proteins and polysaccharides were trapped in large complexes coated with sodium dodecyl sulfate. The addition of PVP into CTAB based extractions to absorb phenolics, preventing their oxidation that renders DNA unusable for downstream application, has been used successfully for

recalcitrant plant species [9,19,20], typically at a concentration of 1 - 2% (w/v). However, in our protocol, the use of 4% PVP proved effective in complete elimination of polyphenols and resulted in white to clear DNA pellets. This study contradicts reports by Healey et al. [21] who found that addition of 4% PVP to the traditional CTAB extraction method failed to isolate any useable DNA from *Corymbia citriodora* subsp. Variegate and upon precipitation, a minute brown pellet was observed. EDTA is a chelating agent; it chelates Mg^{++} ions necessary for DNase activity. In the present protocol, EDTA was increased to 0.2 M from the standard 0.02 M to and this ensured DNA remained protected from the DNase enzyme.

Denaturation and removal of protein are very important to avoid its interference with DNA. Thus chloroform: isoamyl alcohol (24:1) was used for denaturing proteins from DNA allowing only DNA in the supernatant after centrifugation step [22]. In the present study, supernatant of some samples had some greenish to brownish color, and this was eliminated by repeating chloroform-isoamyl alcohol step. DNA was precipitated by using absolute ethanol and ammonium acetate. In presence of water, ammonium acetate donates NH_4^+ to DNA strand and interacts with negative charges of the phosphate group of DNA to form complex to reduce repulsion of both strands (DNA strand negative charge) and help to obtain DNA in an intact form. The ammonium acetate removed the enlacing polysaccharides of nucleic acids during DNA precipitation. Finally, the DNA pellet was washed with 70% ethanol to remove salts, a white color jelly like DNA was observed at bottom of eppendorf tube. Addition of RNase A (10 mg/ml) after tissue homogenization step as described by Chen et al. [23] was tested but did not eliminate RNA. In this protocol, RNase was used at the final step and was found to be effective in complete elimination of RNA.

Plant DNA free of inhibitory metabolites, is required for PCR and other PCR-based techniques such as SSR [24]. To evaluate the suitability of the isolated DNA in downstream applications, we subjected the total DNA to amplification through PCR using SSR markers and virus-specific primers for EACMV. The DNA extracted by this method (CTAB-SDS) yielded good quality bands which were reproducible and scorable proving its suitability for PCR applications using SSR gene marker and also for detection of EACMV (Figs. 2 and 3). This is an indication that the DNA was free from contaminating polysaccharides, polyphenols and ethanol used in washing the pellet [3,12]. PCR analysis with DNA obtained by CTAB and commercial kit based methods was not consistent and the staining with ethidium bromide gave opaque bands indicating a very low concentration of amplified products (Fig. 2). The inability to detect presence of EACMV in DNA extracted from symptomatic plants using CTAB method through PCR could be attributed to either presence of inhibitors in the extracted DNA or low viral titers [25] in the infected plants, making the method unreliable in detecting viruses in asymptomatic plants.

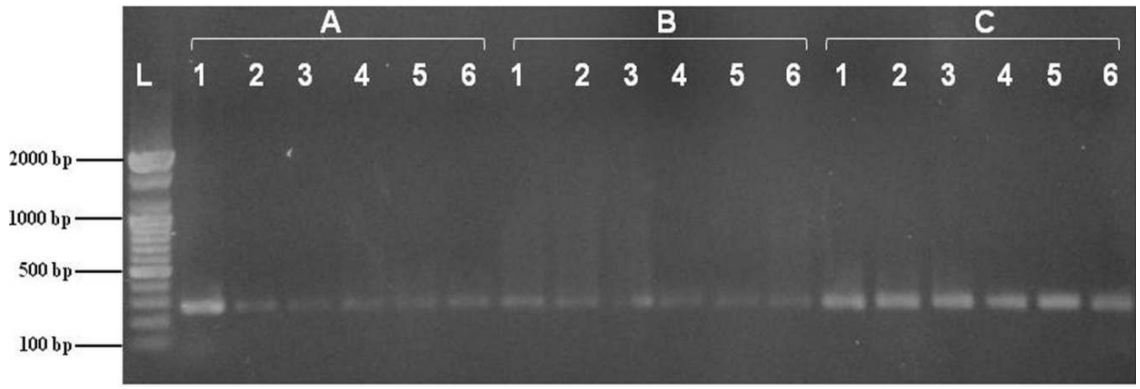

Fig. 2. Electrophoretic profile of SSR amplification (270 bp) using primer SRY9 on 1.5% agarose gel. Lane L - 100 bp molecular weight marker (Bioneer) and Lanes 1 – 6 represents DNA samples isolated from cultivars Kibandameno, Seveu, Mkombozi, TMS60444, TME14 and TME419, respectively. A, B and C represent DNA extracted using DNeasy™ plant mini kit, CTAB and modified CTAB-SDS protocols, respectively

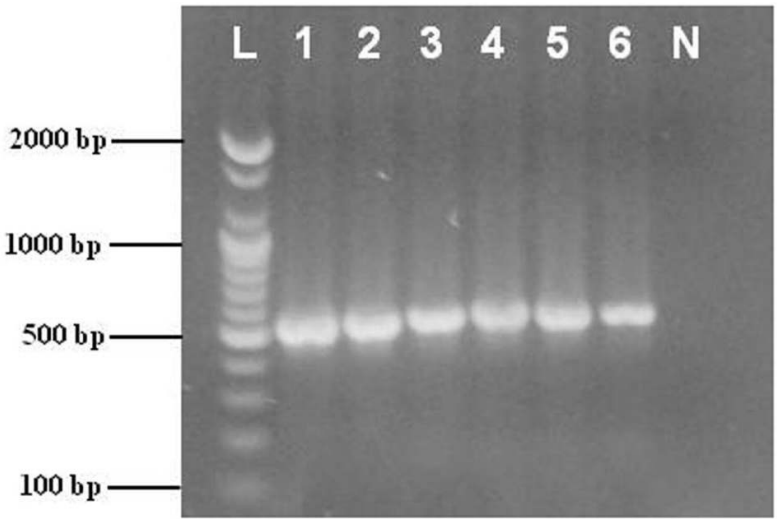

Fig. 3. Agarose gel electrophoresis showing amplification (550 bp) of East Africa cassava mosaic virus using EAB555 primer in DNA samples extracted using CTAB-SDS method. Lane L - 100 bp molecular weight marker (Bioneer) and Lanes 1 – 6 represents DNA samples isolated from cultivars Kibandameno, Seveu, Mkombozi, TMS60444, TME14 and TME419, respectively

In our proposed protocol, the following modifications were considered: elimination of lyophilization of tissues and use of liquid nitrogen, applying PVP into the extraction buffer, addition of SDS and concentration of DNA in solution by a combination of ethanol and ammonium acetate for precipitation. Previous reports on high-quality plant DNA extraction methods [26,27,28,29] used liquid nitrogen, lyophilization, alternating cold (about −80℃), enzymatic digestion for grinding and/or rupturing of the cell and nuclear walls. However, our modified DNA extraction protocol which neither utilized liquid nitrogen, lyophilization, alternating cold (about −80℃), nor enzymatic digestion for grinding and/or rupturing of the cell and nuclear walls, resulted in high quality and quantity DNA. The modified CTAB method described in the present study is already adopted in the experimental laboratory for routine use in molecular biology and it could be suitable for frontier research in developing counties of Africa.

4. CONCLUSIONS

In conclusion, the study has shown that the improvement of CTAB method resulted to a rapid, efficient and reliable DNA extraction method for cassava tissues, rich in polysaccharides and polyphenols. In contrast to the other methods tested, the DNA prepared from various cassava cultivars by this method was intact and of high-quality and -quantity. Our method allows for the efficient molecular analysis, including PCR amplification by SSR markers and virus detection and the DNA isolated by this procedure could be applied for other molecular biology techniques and functional genomic studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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