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Full Length Research Paper

An improved method of DNA extraction from plants for pathogen detection and genotyping by polymerase chain reaction

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Polymerase chain reaction (PCR)-based applications in plant molecular biology and molecular diagnostics for plant pathogens require good quality DNA for reliable and reproducible results. Leaf tissue is often the choice for DNA extraction, but the use of other sources such as tubers, stems, or seeds, is not uncommon. The extraction of DNA from different tissue sources often requires different protocols. In this study, a simple protocol was established for the extraction of DNA from leaves, tubers, stems, seeds and even fungal mycelia. The protocol is simple and suitable for high-throughput DNA extraction using automated tissue grinders. It yielded large quantities of DNA (0.4 µg to 2 mg DNA from 100 mg tissue) of high quality from seeds of maize, soybean, and cowpea, tubers of yam, tuberous roots of cassava, and leaf tissues of banana and plantain, yam, cassava, maize, okra, mango, and other species. DNA was successfully used for the detection of fungal and viral pathogens and the genotyping of yam and cassava by PCR.

Key words: DNA isolation, plant tissues, PCR amplification, pathogen detection, high throughput DNA extraction.

INTRODUCTION

Extraction of a suitable quantity and quality of DNA is a critical step in molecular applications, such as polymerase chain reaction (PCR), sequencing, and cloning and restriction analysis. Several protocols have been established for DNA extraction, some of which are applicable on wide range of materials and some are specific to a particular tissue type (Weishing et al., 1995; Amani et al., 2011; Carrier et al., 2011; Pervaiz et al., 2011; Akkurt, 2012). Generally, freshly harvested leaves are preferred for extraction of DNA (Zhang and Stewart, 2000). In addition, extraction of DNA from preserved leaf tissues, seeds, stems, and tubers is not uncommon. However, the presence of polysaccharides, tannins and other metabolites with high affinity to DNA and protein in these tissues makes it difficult to extract good quality DNA for further utilization in molecular techniques. Polysaccharides inhibit the action of *Taq* polymerase, thus affecting the PCR reaction process (Rogers and Bendich, 1985; Wulff et al., 2002). Similarly, mucilage, a highly viscous secondary metabolite composed of a polar polymer of glycoprotein and present in tubers, seeds, and stems, also co-precipitates with DNA and inhibits the action of *Taq* polymerase (Jose and Usha, 2000; Ghosh et al., 2009). The presence of mucilage also hinders the accurate pipetting of DNA, leading to volumetric errors (Ghosh et al., 2009).

A number of studies have been carried out in the past to establish protocols for the extraction of good quality

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Abbreviations: LTP, Low throughput; **HTP,** high throughput; **DEB,** DNA extraction buffer; **PCR,** polymerase chain reaction; **ITS,** internal transcribed spacer; **BSV,** *Banana streak virus*; **ACMV,** *African cassava mosaic virus*; **EACMCV,** *East Africa cassava mosaic Cameroon virus*; **MSV,** *Maize streak virus*; **SSR,** simple sequence repeat; **PEG**, polyethylene glycol.

DNA from seeds (Kang et al., 1998), tubers (Wulff et al., 2002), mycelia (Chi et al., 2009), and other tissues. In this study, several available protocols were evaluated to extract good quality DNA from a wide array of tissues, with the aim of developing a technically simple, rapid, and cost-effective protocol for manual and high-throughput DNA extraction using automated tissue grinders.

MATERIALS AND METHODS

Tissue materials and PCR targets

Seeds of cowpea, soybean, and maize, tubers of yam (*Dioscorea* sp.), stems and tuberous roots of cassava, and leaves of mango, banana, maize, and okra were collected from the experimental fields in IITA, Ibadan, Nigeria. In addition, mycelia of pure cultures of *Colletotrichum gloeosporioides,* isolated from anthracnose-affected yam leaves and *Cercospora* sp. isolated from gray leaf spot-affected maize leaves were also included. For genotyping by PCR, DNA extracted from leaves (banana, cassava, okra, and mango), tubers (yam and cassava), stems (cassava), and seeds (soybean, cowpea, and maize) of all species were tested using oligonucleotide primers specific to nuclear ribosomal DNA or simple sequence repeat (SSR) markers (Table 1).

For pathogen diagnostics by PCR, DNA extracted from cassava leaves, stems, and tubers, both healthy and damaged by cassava mosaic disease (CMD), was tested for *African cassava mosaic virus* (ACMV) or *East African cassava mosaic Cameroon virus* (EACMCV). Leaves of maize and banana/plantain affected by streak disease were tested for *Maize streak virus* (MSV) or *Banana streak virus* (BSV). DNA extracted from pure mycelia of *C. gloeosporioides* and *Cercospora* sp., from maize was used for the amplification of nuclear ribosomal DNA for fungal genotyping. DNA was also extracted from FTA® Classic Cards (Whatman International Inc.) (Borman et al., 2006), a paper-based system designed to fix and store nucleic acids directly from fresh tissues pressed into the FTA® paper. Leaf sap of CMD-affected cassava leaves was applied on FTA® cards and allowed to dry at room temperature. The cards were stored in envelopes at room temperature until used for DNA extraction and subsequent analysis for ACMV/EACMCV by PCR.

DNA extraction protocol

Reference was made to several DNA extraction protocols to develop a high-throughput, rapid, and cost-effective method for large-scale DNA extraction from a wide range of tissue materials. The method described here was developed based on the protocols described in Kang et al. (1988) and Wuff et al. (2002) by introducing the following modifications:

1. Instead of samples being freeze-dried, seeds or tubers were heat treated at 65°C for 30 min;
2. The phenol: chloroform: isoamyl alcohol (25:24:1) step was removed;
3. CTAB was replaced by SDS as the detergent in the extraction buffer and
4. PEG (40,000 mol. wt.) was added in the extraction buffer.

Tissues were homogenized with sterile pestles and mortars or in 1.5 ml microfuge tubes using handheld micro tube pestles for manual extraction or by using GenoGrinder2000 for high-throughput (HTP) extraction (Bhattacharjee et al., 2009). For DNA extraction by HTP, leaves, stems, or mycelia were stored overnight at –80°C; seeds and tubers were treated at 65°C for 30 min before being ground. About 100 mg of tissue (leaves, tubers, stems, seeds, or mycelia) was ground following both the manual extraction and HTP methods, and samples were incubated at 65°C for 30 min in a water bath after the addition of 500 µL of DNA extraction buffer (DEB; 100 mM Tris, pH 8, 10 mM EDTA, 1% SDS, 2% PVP, 1 M NaCl, and 1% β-mercaptoethanol) supplemented with 0.05 mg/ml proteinase K and 4% (w/v) PEG (mol. wt. 40 000). The samples were then subjected to centrifugation at 13 000 rpm for 10 min in a tabletop centrifuge and the supernatant was collected into fresh microtubes. To each sample vial two-thirds volume of isopropanol (v/v) was added and incubated at –20°C for 1 h for DNA precipitation. The samples were centrifuged at 13 000 rpm for 10 min and the supernatant was discarded to obtain the DNA pellet. Each DNA pellet was further washed with 500 µL of 70% ethanol and centrifuged at 13 000 rpm for 5 min. The supernatant was discarded and traces of ethanol were removed by air drying the DNA pellet for about 15 min at room temperature. The DNA was re-suspended in 100 µL of TE buffer (10 mM Tris, pH 8, and 1 mM EDTA), treated with RNAase A (10 µg/100 µL of DNA sample), and stored at –20°C until further use. DNA was quantified in the NanoDrop ND1000 (NanoDrop Technologies) spectrophotometer by taking readings at 260 and 280 nm, following the manufacturer's instructions. DNA quality was assessed by electrophoresis of an aliquot of 1 µL DNA in an 1% agarose gel using TAE buffer, as described in Sambrook and Russell (2001).

DNA amplification by PCR

The DNA extracted by manual extraction and HTP methods from various sources were tested by PCR amplification using different primer sets listed in the Table 1. PCR for pathogen detection was performed in a total volume of 25 µL, consisting 5.0 µL of 10x PCR buffer (Promega, Madison, WI, USA), 0.5 µL of 10 mM dNTPs, 0.5 µL of 10 pmol of each primer, 1.5 µL of 25 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Promega), and 10 ng of template DNA. The thermal cycle conditions for PCR using various primer sets are given in Table 1. The amplified products were electrophoresed on 1% TAE agarose gels containing 0.5 mg/ml ethidium bromide, and visualized on a UV transilluminator (Sambrook and Russell, 2001).

For genotyping by PCR, DNA extracted from leaves and tubers of cassava were subjected to PCR amplification using SSR markers (Table 1). The total volume of PCR reaction was 10 µL, containing 10 ng of genomic DNA, 1.0 µL of 10× reaction buffers, 0.3 µL of 50 mM $MgCl_2$, 0.8 µL of 2.5 mM dNTPs, 2 µL of each primer and 0.06 U of Platinum *Taq* polymerase (Invitrogen). The PCR products were electrophoresed on 1.2% agarose gel containing 0.5 mg/ml ethidium bromide, and visualized on a UV transilluminator. PCR was also performed by serially diluting 1 µg/ml DNA from $1:10^2$ to $1:10^{10}$ (v/v) in TE buffer, and 2 µL of it was used in PCR to assess the end-point detection of pathogens as a measure to assess the sensitivity of PCR.

RESULTS AND DISCUSSION

DNA quantity and quality

To date, many simplified DNA extraction methods have been developed for fungi and plant tissues, each having its own advantages and disadvantages (Cenis, 1992; Thomson and Henry, 1995; Liu et al., 2000; Cassago et al., 2002; Guo et al., 2005; Chi et al., 2009). In this study, a DNA extraction protocol was standardized, which is a modification of the methods reported by Kang et al. (1998) and Wulff et al. (2002). The protocol developed

Table 1. Details of PCR targets, oligonucleotide primers and PCR conditions used in the study.

Target	Species*	Amplicon size (bp)	Primer name and sequence (5' to 3')	Thermal cycling conditions	Reference
Nuclear ribosomal rDNA (universal)	All tested in present study	550	ITS1:TCCGTAGGTGAACCTGCGG ITS4: TCCTCCGCTTATTGATATGC	One cycle of 94°C/5 min ; 35 cycles of 94°C/30 s, 55°C/1 min., 72°C/1.5 min ; one cycle of 72°C/7 min; and hold at 4°C.	White et al 1990
Nuclear ribosomal rDNA (species-specific)	Yam	500	ITS1:TCCGTAGGTGAACCTGCGG YA(I)F:CCCTTTGTGAACATACC	One cycle of 94°C/5 min ; 35 cycles of 94°C/30 s, 55°C/1 min., 72 ºC/1.5 min ; one cycle of 72°C/7 min; and hold at 4°C.	ITS1 (White et al 1990); YA(I)F (Sharma et al 2009)
Maize streak virus (MSV)	Maize	1400	MSV215-234: CCAAAKDTCAGCTCCTCCG MSV1770-1792: TTGGVCCGMVGATGTASAG	One cycle of 94°C/5 min ; 35 cycles of 94°C/30 s, 52°C/1 min., 72°C/1.5 min ; one cycle of 72 ºC/7 min; and hold at 4°C.	Palmer and Rybicki 2001
Banana streak virus (BSV)	Banana	520	BadnaFP: ATGCCITTYGGIITIAARAAYGCICC Badna RP:CCAYTTRCAIACISCICCCCAICC	One cycle of 94°C/5 min ; 35 cycles of 94ºC/30 s, 50ºC/1 min., 72°C/1.5 min ; one cycle of 72°C/7 min; and hold at 4°C.	Yang et al 2003
African cassava mosaic virus (ACMV) / *East African cassava mosaic Cameroon virus* (EACMCV)	Cassava	ACMV-380 EACMCV- 650	OjaRep-F: CRTCAATGACGTTGTACCA EACMVRep-R: GGTTTGCAGAGAACTACATC ACMVRep-R: CAGCGGMAGTAAGTCMGA	One cycle of 94°C/5 min, 52°C/2min, 72°C/3min; 35 cycles of 94°C/30 s, 52°C/1 min., 72°C/1.5 min ; one cycle of 72ºC/7 min; and hold at 4 ºC.	Alabi et al 2008
EST SSR	Cassava	230, 280, 300	AGC-129 TCT140 AGC163	One cycle of 94°C/1 min, 52°C/2min, 72°C/3min; 35 cycles of 94°C/30 s, 52 ºC/2 min., 72°C/1.33 min; one cycle of 72°C/7 min; and hold at 4°C.	de Bang et al 2010
EST SSR	Yam	210, 280, 400	Dab2C05 (F and R)- CCCATGCTTGTAGTTGT-F TGCTCACCTCTTTACTTG-R DaIA01 (F&R)- AACTATAATCGGCCAGAGG-F TGTTGGAAGCATAGAGAATT-R	One cycle of 94°C/4 min; 34 cycles of 94°C/30 s, 55°C/30 s, 72C/1 min; one cycle of 72°C/7 min; and hold at 4°C.	Tostain et al 2006.

Table 2. Concentration of DNA extracted using three different protocols.

Sample	This paper			As per Kang et al. (1995)			As per Wulff et al. (2002)		
	*DNA (ng/µl)	$A_{260/280}$	$A_{260/230}$	DNA (ng/µl)	$A_{260/280}$	$A_{260/230}$	DNA (ng/µl)	$A_{260/280}$	$A_{260/230}$
Yam tuber	1147.9 ±117.0	1.8 ±0.0	0.5 ±0.4	63.4 ±9.1	1.1 ±0.1	0.3 ±0.0	222.3 ±0.9	1.3 ±0.0	0.4 ±0.0
Cassava stem	506.5 ±36.2	1.7 ± 0.2	0.4 ±0.3	38.7 ±11.3	1.3 ±0.2	-12.3 ±21.7	147.4 ±4.5	1.5 ±0.0	0.5 ±0.0
Cowpea seed	4538.4 ±232.7	1.7 ±0.2	0.7 ±0.6	1154.7 ±73.3	0.7 ±0.0	0.3 ±0.0	27.0 ±0.5	0.9 ±0.0	0.1 ±0.0
Soybean seed	3896.6 ±522.1	1.6 ±0.4	0.6 ±0.5	499.9 ±33.4	0.9 ±0.0	2.2 ±0.8	1289.9 ±170.2	1.1 ±0.0	0.4 ±0.0
Maize leaf	870.3 ±32.4	1.8 ±0.1	0.9 ±0.1	39.3 ±1.7	0.9 ±0.0	0.2 ±0.0	67.8 ±1.4	1.1 ±0.0	0.2 ±0.0
Banana leaf	4417.0 ±334.3	1.9 ±0.1	0.7 ±0.9	nt	nt	nt	nt	nt	nt
Mango leaf	917.4 ±30.0	1.9 ±0.0	0.3 ±0.3	nt	nt	nt	nt	nt	nt
Okara leaf	851.9 ±28.3	1.8 ±0.1	1.0 ±0.1	nt	nt	nt	nt	nt	nt
Cg* *(mycelia)*	1860.9 ±548.9	1.7 ±0.2	0.4 ±0.4	nt	nt	nt	nt	nt	nt

*Concentration estimated using NanoDrop Spectrophotometer ND-1000; nt, not tested; Cg, *Colletotrichum gloeosporioides.*

has several advantages:

a. The number of steps is minimal and thus large numbers of samples can be processed/day/person;
b. It can be efficiently used to obtain a sufficient amount of DNA from 100 mg of seed, tuber, or leaf tissues;
c. The protocol does not use liquid nitrogen and toxic chemicals such as phenol;
d. It is cost-effective protocol ($2 to $3/sample depending on manual extraction or HTP is used; and
e. Facilitates good quality DNA from different plant tissues. This protocol was successfully used to isolate DNA from several plant species, including banana, yam, okra, banana, mango and even fungal mycelia, from which genomic DNA isolation is difficult due to the presence of a high amount of mucilage, polyphenols and proteins (Cassago et al., 2002).

This protocol advocates the incubation of seeds/tubers in hot water for 30 min, which helps in softening the tissues for easy grinding (Kang et al., 1998); detergent, CTAB was been replaced with SDS to obtain good quality DNA (Edwards et al., 1991; Keb Llanes et al., 2002; Matasyoh et al., 2008). Generally, CTAB is used to avoid co-purifying polysaccharides from plant tissues; SDS breaks up the lipids in the membranes. Since seed and tuber samples carry high amounts of polysaccharides and mucilage, SDS was used in the extraction buffer. The extraction buffer contains proteinase K and PEG which helped in removing proteins and phenolic components, thus excluding from the protocol the organic solvents (such as phenol and chloroform).

The results from NanoDrop spectrophotometer showed that the protocol described in this study yielded a good quantity and quality of DNA (Table 2, Figure 1A). The concentration of DNA ranged between 506.5 and 4538.4 ng/100 mg of seeds and tubers; from 13.3 to 1176.24 ng of DNA/ 50 mg of mycelia; and from 766.98 to 1581.92 ng/ 100 mg of seeds (Table 2). The A260/280 ratio was 1.23 to 2.26 for DNA extracted from mycelia, and 1.61 to 1.92 for DNA extracted from seeds. It was observed that the addition of PEG in the extraction buffer increased the quality of DNA; this may be due to the effective removal of polysaccharides present in tissue samples. PEG is regularly used for tissue samples which are known to contain high amounts of polysaccharides or mucilage (Sharma et al., 2008).

PCR amplification

The DNA extracted from anthracnose-affected yam leaf samples, uninfected controls, and purified cultures of *C. gloeosporioides* using both manual extraction and HTP methods was amplified using universal rDNA primers and primers specific to *C. gloeosporioides*. Amplification of 550 bp DNA with universal rDNA primers and 500 bp DNA from the anthracnose-affected leaves and a pure culture of *gloeosporioides* confirmed that the quality of DNA extracted using the protocol was suitable for PCR amplification (Figures 1B and 2A).

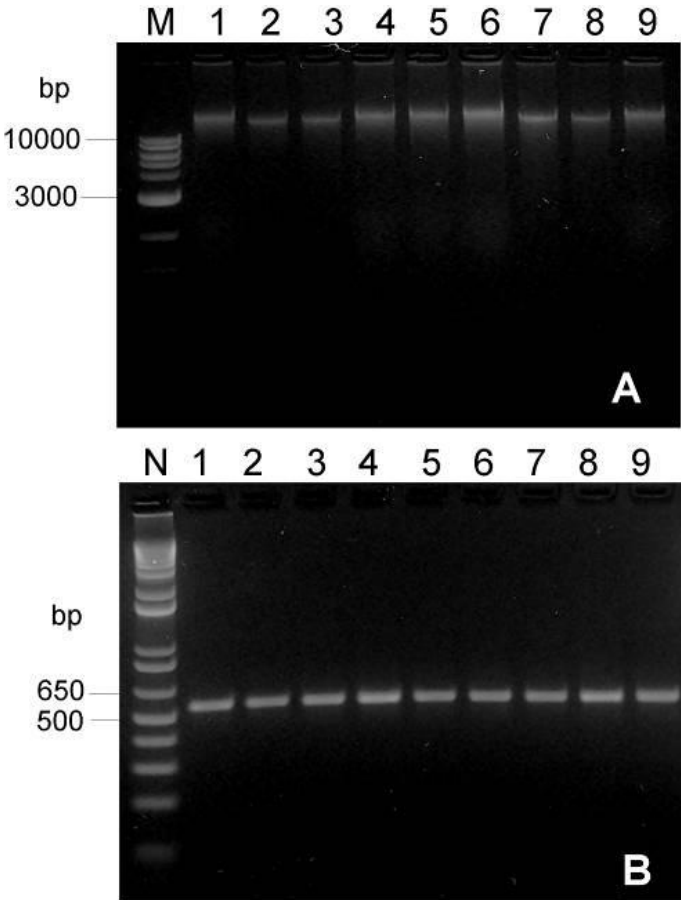

Figure 1. Resolution of DNA (A) and nuclear rDNA ITS segment amplified by ITS-1/ITS-4 primers in PCR (B). Lanes 1, Yam tuber; 2, Cassava tuber; 3, Cowpea seeds; 4, Soybean seeds; 5, Maize seeds; 6, Banana leaf; 7, Mango leaf; 8, Okra leaf; 9, *mycelia of C. gloeosporioides;* Lane M, 1 kb DNA marker (New England Biolab); and Lane N, 1kb plus DNA standard (Invitrogen). Position of molecular weight markers indicated in base pairs (bp).

DNA extracted from BSV-infected leaves of banana was amplified by ITS (Figure 2B; Lanes 1 to 2) used as controls and BSV-specific primers (Figure 2B; Lanes 3 to 5). The BSV-specific band was also observed with an asymptomatic banana leaf which could correspond to the BSV genome integrated in the host genome. The DNA samples extracted from healthy and infected leaves and tubers of cassava were amplified by ITS primers resulting in a 550 bp amplicon. ACMV/EACMCV-specific primers amplified a 390 bp amplicon specific to ACMV in CMD-affected cassava leaf, but not in healthy cassava. EACMCV specific primers did not result in amplification, indicating that the sample was free from EACMCV (Figure 2C). MSV specific amplicon resulted in PCR of MSV infected maize (Figure 2D).

The DNA extracted from FTA^{R} cards spotted with cassava leaf extract resulted in the amplification of a ~390 bp product of ACMV and a ~650bp product of EACMCV (Figure 2E), confirming the suitability of the protocol for extracting DNA even from FTA^{TM} cards, which are used for sample preservation during field surveys and subsequent laboratory diagnosis of pathogens (Borman et al., 2006). Pathogens detection in serially diluted DNA differed between the samples (Table 3) (Figure 3). For instance, ACMV was detected up to 10^{-6} dilution of 1 µg/µL DNA extracted from leaves, but up to 10^{-3} and 10^{-5} dilution in DNA extracted from stems and tubers, respectively (Figure 3). These results could be a reflection of variable concentration of ACMV in different tissues of cassava. Interestingly, PCR amplification of rDNA ITS regions using the DNA extracted from yam and banana leaves resulted from 10^{-4} DNA dilution (Table 3). High concentration of leaf polyphenols in yam and banana leaves might be interfering with PCR reactions at low dilutions ($<10^{-4}$). This result suggests that appropriate DNA dilution is critical to successful PCR, particularly

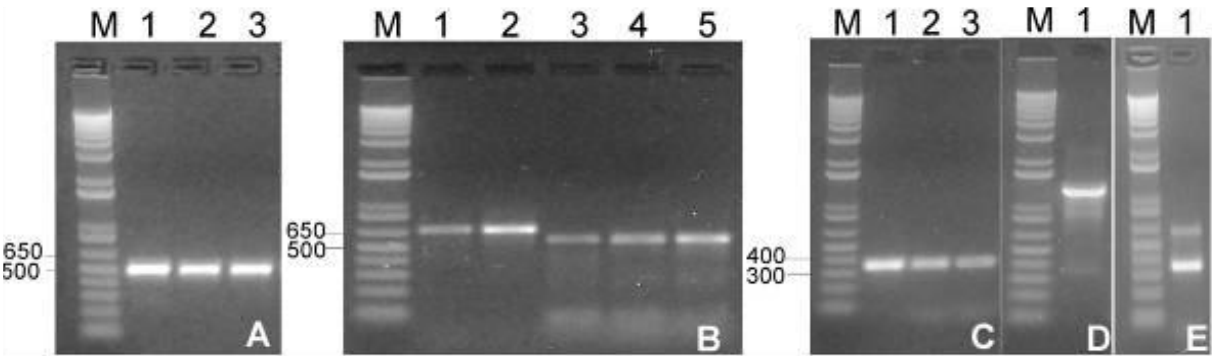

Figure 2. A. Detection of *C. gloeosporioides* in yam leaf DNA using ITS1 and YA(I)F (Lanes 1 to 3); B. Amplification of banana rDNA using ITS1/ITS4 primers (Lanes 1 and 2), and *Banana streak virus* (Lanes 3 to 5); C. Detection of *African cassava mosaic virus* (ACMV) in cassava mosaic disease-affected cassava leaf (Lane 1), stem (Lane 2) and tuber (Lane 3); D. Detection of *Maize streak virus* in the virus infected maize leaf using; E. Detection of ACMV and *East African cassava mosaic virus* in DNA extracted from FTA® Classic card spotted with the infected cassava leaf sap. Lane M, 1 kb DNA marker (NewEngland Biolab). Position of molecular weight markers indicated in base pairs (bp).

Table 3. Serial dilutions PCR using specific primer for pathogens.

Target*	DNA dilution**								
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
ITS amplification using ITS1/ITS4 primers in *C. gloeosporioides* mycelia	+	+	+	+	+	+	nt	nt	nt
ITS amplification in yam leaf using ITS1/ ITS4	-	-	+	+	+	+	nt	nt	nt
ITS amplification in maize leaf using ITS1/ ITS4	+	+	+	+	+	-	-	-	-
ITS amplification in maize seed using ITS1/ ITS4	+	+	+	+	+	+	+	-	-
ITS amplification in cassava leaf using ITS1/ ITS4	+	+	+	+	+	+	-	-	-
ITS amplification in cassava tuber using ITS1/ ITS4	+	+	+	+	+	-	-	-	-
Detection of BSV in banana leaf	-	-	+	+	+	nt	nt	nt	nt
Detection of MSV in maize leaf MSV infected leaf using MSV	+	+	+	+	+	nt	nt	nt	nt
Detection of ACMV/EACMCV in cassava leaves	+	+	+	+	+	nt	nt	nt	nt
Detection of ACMV/EACMCV in cassava stems	+	+	-	-	-	nt	nt	nt	nt
Detection of ACMV/EACMCV in cassava tubers	+	+	+	+	-	nt	nt	nt	nt

*Primer details and PCR conditions for each target is provided in Table 1. **Starting DNA concentration, 1 µg/µl; +, PCR amplification; -, no amplification; nt, not tested.

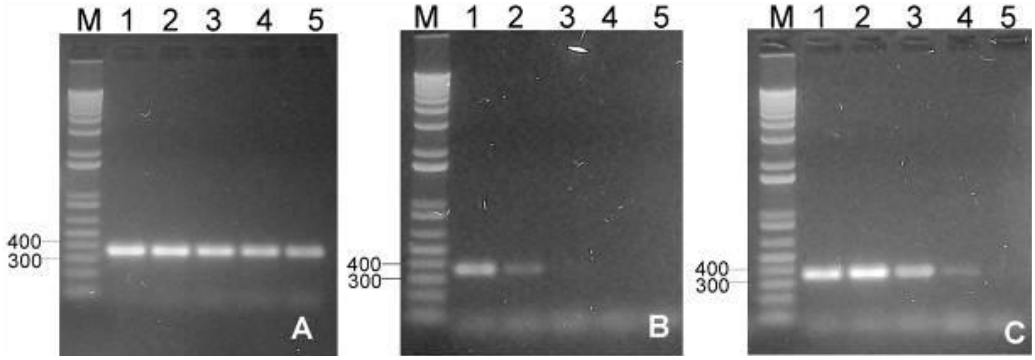

Figure 3. Detection of *African cassava mosaic virus* (ACMV) in DNA extracted from cassava leaves (A), stems (B) and roots (C). Lanes 1 to 5, DNA dilution from 10^{-2} to 10^{-6}. AMCV was detected up to 10^{-6} dilution in leaves (A), up to 10^{-3} dilution in stems (B) and up to 10^{-5} dilution in tubers (C). Lane M, 1 kb DNA marker (New England Biolab). Position of molecular weight markers indicated in base pairs (bp).

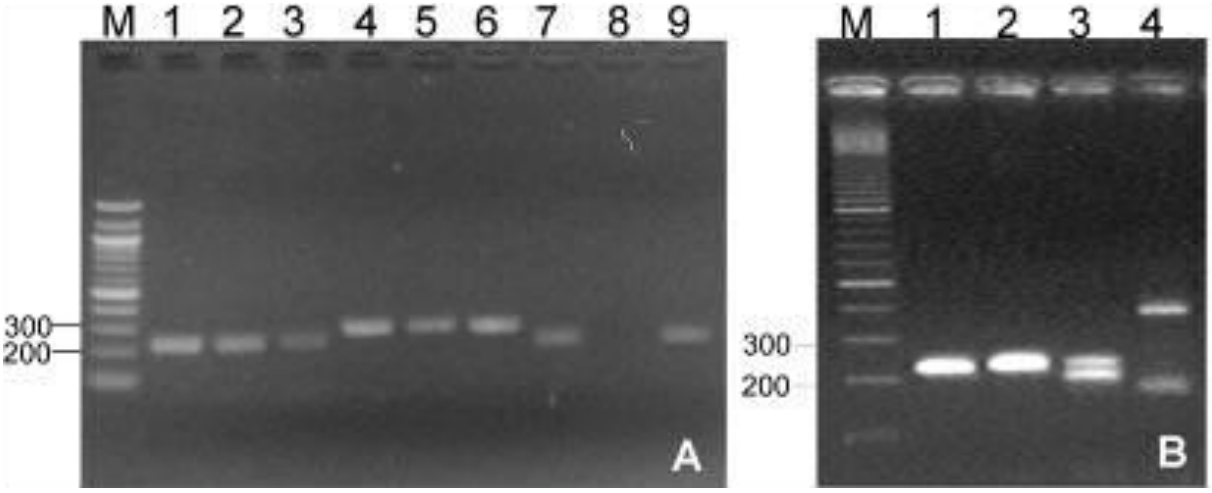

Figure 4. A. Amplification of cassava genomic DNA by PCR using SSR primers. Lanes 1, cassava leaf; 2. cassava stem; 3. cassava tuber; 4. cassava leaf; 5. cassava stem; 6. cassava tuber; 7. cassava leaf; 8. cassava stem; 9. cassava tuber. Lane 1-3 using SSR marker AGC129 forward and reverse; Lane 4-6 using SSR marker TCT140 forward and reverse; Lane 7-9 using SSR marker AGC163 forward and reverse. Lane M, 100bp DNA standard (NewEngland Biolab). B. Amplification of yam genomic DNA by PCR using SSR primers. Lanes: 1, yam leaf; 2, yam tuber; 3, yam leaf; 4, yam tuber. Lane 1 to 2 using SSR markerDa1A01 forward and reverse; Lane 3-4 using SSR marker Dab2C05 forward and reverse. Lane M, 100bp DNA standard (Promega). Position of molecular weight markers indicated in base pair (bp).

when plant samples rich in polyphenols are used as source materials. The SSR primers used for genotyping using DNA extracted from leaves and stems of cassava and leaves and tubers of yam resulted in amplification (Figure 4). By validating the improved DNA extraction protocol on diverse samples, this study demonstrated the suitability of the technique as a simple and cheaper alternative to extract DNA for PCR applications from many plant species and tissue sources.

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