

Full Length Research Paper

Optimised cetyltrimethylammonium bromide (CTAB) DNA extraction method of plant leaf with high polysaccharide and polyphenolic compounds for downstream reliable molecular analyses

Turaki A. A.^{1,2,3*}, Ahmad B.³, Magaji U. F.³, Abdulrazak U. K.³, Yusuf B. A.³ and Hamza A. B.^{1,2}

¹Natural Resources Institute, University of Greenwich, UK.

²Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria.

³Federal University of Birnin Kebbi, Kebbi State, Nigeria.

Received 14 February, 2017; Accepted 28 April, 2017

Yam (*Dioscorea* species) and banana (*Musa* species) leaf sample contain high levels of polysaccharide and polyphenolic compounds. Extraction of good quality DNA from such leaves is usually problematic. Therefore, there is a need to extract good quality DNA in order to perform downstream DNA analysis, especially for the detection of viruses due to their low titration in some infected leaves. The cetyltrimethylammonium bromide (CTAB) DNA extraction method was selected in order to optimise DNA extraction from plant material with high level of polysaccharides. Four steps of the Lodhi et al. (1994) method were modified to be user friendly in less resource laboratories of developing countries. Yield and purity of the extracted DNAs were quantified using a NanoDrop 2000 spectrophotometer and by migration on an agarose gel after polymerase chain reaction (PCR) amplification. The results of the DNA yields and purity were in the range of 287.40 ± 2.23 to 424.95 ± 1.85 ng/ul and 2.10 ± 0.05 to 2.19 ± 0.04 , respectively. Modification was found to yield DNA of reasonable quantity, quality and purity. Furthermore, the method can be used in a laboratory with less sophisticated waste disposal system.

Key words: Deoxyribonucleic acid (DNA), cetyltrimethyl ammonium bromide (CTAB), yam, banana, tomato.

INTRODUCTION

Yam (*Dioscorea* species) leaf sample contain high levels of polysaccharide and polyphenolic compounds. The presences of these compounds in yam leaf tissue often affect the isolation of good quality DNA (Kenyon et al.,

2008). This has presented a problem for the downstream molecular analyses of extracted DNAs from yam samples especially in the detection and analysis of yam viruses such as badnaviruses. Moreso, the low virus titre in

*Corresponding author. E-mail: turakialiyu@yahoo.com. Tel: +234(0) 8148534433.

infected yams does not allow the expression of a well-defined symptoms on yams leaves (Seal and Muller, 2007; Seal et al., 2014). Therefore, the need for extracting reasonably pure DNA of both good quality and quantity for the downstream successful DNA-based diagnostic techniques is required. Hence, the aim of this study was to optimise a DNA extraction methodology for plants with high levels of polysaccharide and polyphenolic compounds. Cetyltrimethylammonium bromide (CTAB) DNA extraction method was selected as the method to optimise, as it is generally the method of choice for plant material with high polysaccharides and other inhibitory substances; it has been used successfully for yam in the past (Lebas, 2002; Kenyon et al., 2008) and for other recalcitrant plant species (Csaikl et al., 1998; Michiels et al., 2002; Sharma et al., 2008; Tiwari et al., 2012). Extracted DNA that is pure give good polymerase chain reaction (PCR) products compared to DNA with lower purity which requires several dilutions before it will give a good PCR product. In this study, polymerase chain reaction (PCR) methods were used to evaluate DNA purity by detecting the presence of yam badnavirus sequences on samples known to be badnavirus positive. PCR techniques provide greater sensitivity compared to other methods such as symptom observation and serological methods (Kenyon et al., 2008; Seal et al., 2014).

MATERIALS AND METHODS

Plant

Plant samples used for the modification of the CTAB method and detection of badnaviruses consisted of fresh yam leaf samples obtained from the International Institute of Tropical Agriculture (IITA) (n = 231), dried yam leaf samples collected by Dr. Ed Canning in 1997 (n = 44) during Cameroon survey and Professor Susan Seal in 2001 (n = 7) from CIRAD France, as well as fresh and etiolated leaves of *Dioscorea rotundata*, *Dioscorea alata*, banana and tomato grown in the quarantine glasshouse at Natural Resources Institution, UK. A total of 275 plant samples were used for DNA extraction.

Optimisation of the DNA extraction protocol

The Lodhi et al. (1994) DNA extraction method was modified for the extraction of total DNA from yam leaf material. Banana and tomato leaf tissues were included in the optimisation experiments in order to determine if modifications made were specific improvements for yam tissue alone or modifications to other plant species as well. Banana and yam samples represented plant leaf tissue with high levels of polysaccharides and polyphenols, whereas tomato contains much lower levels of these PCR-inhibitory compounds (Peterson et al., 1997). DNAs extracted from three replicate leaf homogenate samples were used for the comparison of each of the modification steps. These modification steps were incorporated into the Lodhi et al. (1994) method in a stepwise manner and at each step the yield and purity of the extracted DNAs were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). The results of the DNA yields and purity were expressed in mean values \pm standard error (SEM) of DNA in a total elution volume of

100 μ l TE buffer represented in column charts.

Modification steps evaluated

Sodium sulphite and PVP-40 to prevent oxidation of polyphenolic compounds

Lodhi et al. (1994) used PVP-40 (1%) and β -mercaptoethanol (0.2%) in CTAB buffer for the removal of polyphenols. In this study, β -mercaptoethanol (0.2%) was replaced with 1% sodium sulphite (Na_2SO_3) and the PVP-40 concentration was increased to 2%. This replacement was because of the toxicity and difficulty associated with disposing of β -mercaptoethanol waste in developing countries compared to sodium sulphite and PVP-40 waste. Furthermore, the use of sodium sulphite and PVP-40 is more cheaper compared to the use of β -mercaptoethanol. These modification steps were denoted as NS (for the use of 1% Na_2SO_3 with 2% PVP-40) and BM (for the use of 0.2% β -mercaptoethanol with 1% PVP-40).

Cell lysis incubation time for the release of genetic material

The incubation times at elevated temperature are fundamental for the lysis of the cell walls and membranes by detergents (2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl)) and hence this step was optimised. Lodhi et al. (1994) incubated the homogenate for 25 min at 60°C. In this study, homogenates were incubated at 60°C for a range of times (between 25 and 40 min). This variation in time was to find the optimum time required to obtain high yields of extracted DNAs from samples.

Phenol:Chloroform:Isoamyl alcohol step for the removal of proteins

Extraction of DNA involves adding an equal volume of organic solvent to an aqueous solution of tissue homogenate. Centrifugation of the mixture yields a lower organic phase that contains the extracted proteins, and an upper aqueous phase containing DNA. The Lodhi et al. (1994) method uses a chloroform:isoamyl alcohol (CAA 24:1,v/v) extraction step for removal of proteins. A modification step was evaluated by replacing CAA with a phenol:chloroform:isoamyl alcohol (PCAA, 25:24:1, v/v/v) extraction. The mixture of PCAA dissolve more organic compound from the mixture compared to CAA.

Cold isopropanol and sodium chloride for the removal of polysaccharide

Yam contains high levels of polysaccharides (Mumford and Seal, 1997). The Lodhi et al. (1994) method used ethanol with 5 M NaCl to selectively precipitate the DNA away from the polysaccharides. A modification was made in which ethanol was replaced with isopropanol. This was because DNA is less soluble in isopropanol and at low concentration of isopropanol DNA falls out of the solution faster compared to ethanol. The precipitation was performed at -20°C with varying incubation period time intervals (20 to 90 min) in order to select the optimum incubation time in terms of quantity and purity.

Detail steps of the modified Lodhi et al. (1994) CTAB method

- (1) Weigh leaf tissues (~100 mg per sample) and placed in individual polythene bags or in a grinding motor.
- (2) Add 1 ml of warm extraction buffer (2% (w/v) CTAB, 100 mM

Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl) containing freshly added 2% (w/v) polyvinylpyrrolidone-40 (PVP-40) and 1% (w/v) sodium sulfite (Na_2SO_3).

(3) Ground the leaf materials using a small hand roller or pistol

(4) Transfer crude extract (700 μl) into 1.5 ml microcentrifuge tubes

(5) Vortex and incubate in a water bath (60°C, 30 min) for cell lysis.

(6) Add 700 μl phenol:chloroform:isoamylalcohol 25:24:1 (v/v/v) into the incubated extract, vortexed and centrifuged (15000 g, 10 min) in a microcentrifuge tube for first clarification.

(7) Transfer about 700 μl of the aqueous layer carefully into a fresh microcentrifuge tube then add 700 μl chloroform:isoamylalcohol 24:1 (v/v), vortexed and centrifuged (15000 g, 10 min) in a microcentrifuge tube for second clarification.

(8) Transfer about 700 μl of the aqueous layer carefully into a fresh microcentrifuge tube then add 75 μl of 5 M NaCl and 450 μl of cold isopropanol.

(9) Invert the mixture 4 to 5 times then incubate (-20°C, 1 h)

(10) Centrifuge the mixture (4°C, 15000 g, 10 min) to pellet the nucleic acids.

(11) Decanted the supernatant off carefully not to throw out the pellet.

(12) Wash the pellet by adding 500 μl of 70% (v/v) ethanol, and centrifuge (15000 g, 5 min).

(13) Repeat step 12.

(14) Dried the pellet and then re-suspended in 100 μl 1x TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at -20°C until use.

(15) Dilute 1:10 (v/v) extracted DNA in nuclease-free sterile distilled water prior to PCR.

Statistical analyses

The statistical tools used for the analyses of the DNA concentrations and purity results obtained from the DNAs extracted using the CTAB methods were as the following.

t-test

In this study, *t*-test comparing two samples assuming equal variances was used to find out if the null hypothesis is supported.

Analysis of variance (ANOVA)

ANOVA single factor was carried out to determine whether there were significant differences between the DNA concentration and purity obtained in the methods.

The null hypothesis

To determine if there is any significant difference between the DNA concentrations or purity obtained in the DNAs extracted using the modified CTAB method versus those obtained in the Lodhi et al. (1994) method and Lebas (2002).

Comparison of the modified CTAB to other DNA extraction methods

Once all the favoured modifications to the selected Lodhi et al. (1994) method had been made, the resulting revised method was termed the 'modified CTAB' method. DNA yields and purity of this method was then compared with the original method on various leaf materials. DNA extracted from yams (*D. alata* and *D. rotundata*), were compared to DNA extracted from banana and tomato leaves using the three methods of DNA isolation (Lodhi et al., 1994; Lebas,

2002), and newly modified CTAB method. The new modified CTAB method was also compared to the original for the extraction of DNA from leaf tissues that had either been freshly harvested, or had been stored for 24 h at 4°C or room temperature (RT).

Evaluation of extracted DNA as template for restriction digestion PCR and RCA amplification

To assess the effectiveness of the modified method, DNA (5 ng) were digested with restriction enzymes (Figure 4A) and electrophoresed alongside undigested DNA. Furthermore, badnavirus partial RT-RNaseH region (Badna-FP/-RP) primers were used at various DNA dilutions to determine if the expected PCR product (~ 579 bp) could be amplified.

PCR reaction

The PCR protocol was adapted from Yang et al. (2003) and 25 μl reactions were set up containing 1x PCR buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% (v/v) Tween 20) (Thermo Scientific, UK), 1.5 mM MgCl_2 , 0.2 mM of each dNTP (Promega, UK), 0.4 μM of each primer, 1.25 U Super *Taq* polymerase (Thermo Scientific, UK) and 5 μl DNA template. PCR cycling was performed in a GeneAmp® 9700 cycler (Applied Biosystems, UK) programmed as follows: 94°C/2 min initial denaturation, 40 cycles of 94°C/20 s, 55°C/30 s and 72°C/1 min and final cycle of 72°C/10 min.

Gel staining

Agarose gels were stained using 5 μl 'Red Safe™ 20,000x' nucleic acid staining solution (iNtRON Biotechnology Inc., Korea) for 100 ml of warm agarose solution.

HEPES treatment for polysaccharides

A HEPES initial washing step of ground leaf tissue was tried to determine if it was effective at removing polysaccharides from the leaf tissue before extracting total DNAs by the modified CTAB extraction method. Ground fresh leaf tissue (100 mg) was thoroughly mixed with 1 ml of 0.1 M HEPES-buffer pH 8.0 containing (1% (w/v) PVP-40, 1% (w/v) L-ascorbic acid, 2% (v/v) β -mercaptoethanol) and centrifuged (10000 g for 5 min). The supernatant was then discarded before addition of CTAB extraction buffer.

Genomic DNA purification

For the purification of large scale CTAB DNA extractions, pelleted nucleic acids were re-suspended in 1x TE buffer containing 0.7 M NaCl. Once re-suspended, extracts were mixed with an equal volume of equilibration QBT buffer (750 mM NaCl; 50 mM MOPS pH 7.0; 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100; Qiagen, UK). QBT buffer (5 ml) was added to each column (Tip-100\G, Qiagen, UK) and allowed to stand for 5 min for column equilibration. The sample mixtures were then applied to the columns and allowed to stand for 15 to 20 min depending on the follow of the sample through the column. The genomic DNA in the mixture binds to the column resin as the sample passes through the column allowing the RNA, proteins, polysaccharide and low molecular-weight impurities to be removed by a medium-salt wash QC buffer (1 M NaCl; 50 mM MOPS pH 7.0; 15% (v/v) isopropanol, Qiagen, UK). Genomic DNA was then eluted in a high-salt elution QF buffer (1.25 M NaCl; 50 mM Tris-HCl pH 8.5; 15% (v/v) isopropanol, Qiagen, UK) and then

concentrated and desalted by isopropanol precipitation.

Determination of quantity and purity of the extracted DNA

The quantity and purity of the extracted DNAs were analysed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). DNA purity is the ratio of spectrophotometric absorbance of DNA at $\lambda=260$ nm and protein at $\lambda=280$ nm.

RESULTS

Optimisation of DNA extraction protocols

The results obtained with different modification steps are described as the following.

Separation of DNA from polyphenols

The NS step was incorporated into the Lodhi et al. (1994) method to reduce the oxidation of phenolic plant metabolites during DNA extraction. The separation of DNA from phenolic compounds was historically reported as difficult (Ghaffari et al., 2011); however, the inclusion of antioxidants in the extraction buffer assists in overcoming downstream problems associated with the presence of oxidised phenolic compounds in DNA extracts. Results of DNA concentration and purity obtained from the inclusion of NS and BM step in Lodhi et al. (1994) were recorded and their mean values were compared (Figure 1A and B). Although there was a difference between the mean values obtained from both NS and BM inclusions (Figure 1A and B), statistically the *t*-test demonstrated that there was no significant difference ($P>0.05$) between the Lodhi et al. (1994) and modified methods including the NS and BM modification steps. Nevertheless, the utilisation of NS method is considered favourable compared to β -mercaptoethanol because of its toxicity and problems associated the waste disposal of β -mercaptoethanol especially in a less equip laboratory set up.

Release of genetic materials from the cell

To release nuclear material (DNA/RNA) into an extraction buffer is achieved through detergents (CTAB) to lyse cell walls and membranes. In this study, different incubation times (25 to 40 min) at 60°C were evaluated, and the optimum incubation time that yielded the greatest DNA yield was at 60°C for 30 min. The mean values of DNA concentration and purity obtained from the inclusion of NS and BM step and homogenate incubation time for 30 min and 25 min in Lodhi et al. (1994) were recorded and compared (Figure 1A to B). There was a significant difference ($P<0.05$) between the DNA concentrations achieved using the modified method and those by the Lodhi et al. (1994) method. In contrast, there was no significant difference ($P>0.05$) between the DNA purity of the modified method compared to the Lodhi et al. (1994) method. It was observed that incubation for 40 min and above and incubation below 25 min generated lower quantities of DNA and purity (data not shown).

Separation of DNA from proteins

Incubated homogenates were deproteinized using an organic solvent, which reduces the formation of insoluble protein-nucleic acid complex thereby separating the DNA in the aqueous phase and proteins in the organic phase. It is also important to make sure the pH of the organic solvent is >7.8 as a decrease in pH (that is,

more acidic conditions) results in the absence of DNA in the aqueous phase. In this study, CAA was replaced by PCAA as the first clarification in the deproteinization step of the modified method. The results have indicated an increase in the mean values of both DNA purity and quantity as shown in Figure 1A and B. Statistically by the *t*-test, the null hypothesis is therefore rejected as there is a significant difference ($P<0.05$) between the DNA concentration/purity of the modified method and those of Lodhi et al. (1994) method at this modification step. The increase in the mean values of DNA purity for the modified method compared with Lodhi et al. (1994) after PCAA step indicates that PCAA is better at removing proteins from DNA than CAA alone.

Separation of DNA from polysaccharides

Contamination of DNA with polysaccharides is problematic and difficult to eliminate. In this study, extracted DNAs were precipitated through different incubation lengths (20 to 90 min) at -20°C in cold isopropanol together with 5 M NaCl (data not shown). The optimum incubation time for the DNA precipitation was recorded at 1 h producing mean value ratios of $A_{260}/A_{230\text{ nm}}$ above 1.6.

Comparison of the modified CTAB to other DNA extraction methods

The effectiveness of the modified CTAB method (including all favourable modification steps) was compared to Lodhi et al. (1994) using leaf tissues from three different species (banana, *D. rotundata* and tomato) stored at three different conditions (fresh, 4°C/24 h and RT/24 h). From the results, DNAs of high quantities were obtained from fresh leaf samples compared to etiolated leaves, and from the modified method compared to the Lodhi et al. (1994) method (Figure 2A). A significant difference ($P<0.05$) was observed between the DNA concentrations of fresh and etiolated leaf samples of *D. rotundata* and banana (and not for tomato; where $P>0.05$) using both modified method and Lodhi et al. (1994) method (Figure 2B).

Furthermore, the mean values of DNA concentration and purity for the extracted DNAs from four leaf tissues (banana, *D. alata*, *D. rotundata* and tomato) using the modified method were compared with those of the Lodhi et al. (1994) and Lebas (2002) methods as shown in Table 1 and Figure 3A and B. The DNA concentrations obtained from these methods were analysed. Statistically, there was a significant difference ($P<0.05$) between the results obtained with the three different methods for yam *D. rotundata* ($P = 0.0021$), *D. alata* ($P = 0.0035$) and banana ($P = 0.0127$) there was no significant difference ($P>0.05$) for tomato leaves ($P = 0.0701$). The mean value ratios obtained for $A_{260}/A_{230\text{ nm}}$ were above 1.6 indicating low levels of polysaccharide contamination in the extracted DNA, and the $A_{260}/A_{280\text{ nm}}$ ratio were above 1.8 (Table 1) representing low amounts of protein contamination in the extracted DNA.

PCR detection of badnaviruses

The quality of the extracted DNAs were also determined by running undigested and digested total DNA extracts. A perfect migration pattern was observed without any signs of degradation (Figure 4A). PCR of known badnavirus-positive *D. rotundata* leaf samples (fresh, etiolated and dried) were tested for badnavirus in the following dilutions: undiluted, 1:100 dilution and 1:1000 dilution as shown in Figure 4B. The results show that dilution of the DNA sample does affect the outcome of PCR reaction. Diluted DNAs gave intensive PCR bands compared to undiluted DNAs (Figure 4B). Furthermore, ~65% badnavirus PCR success was recorded in undiluted

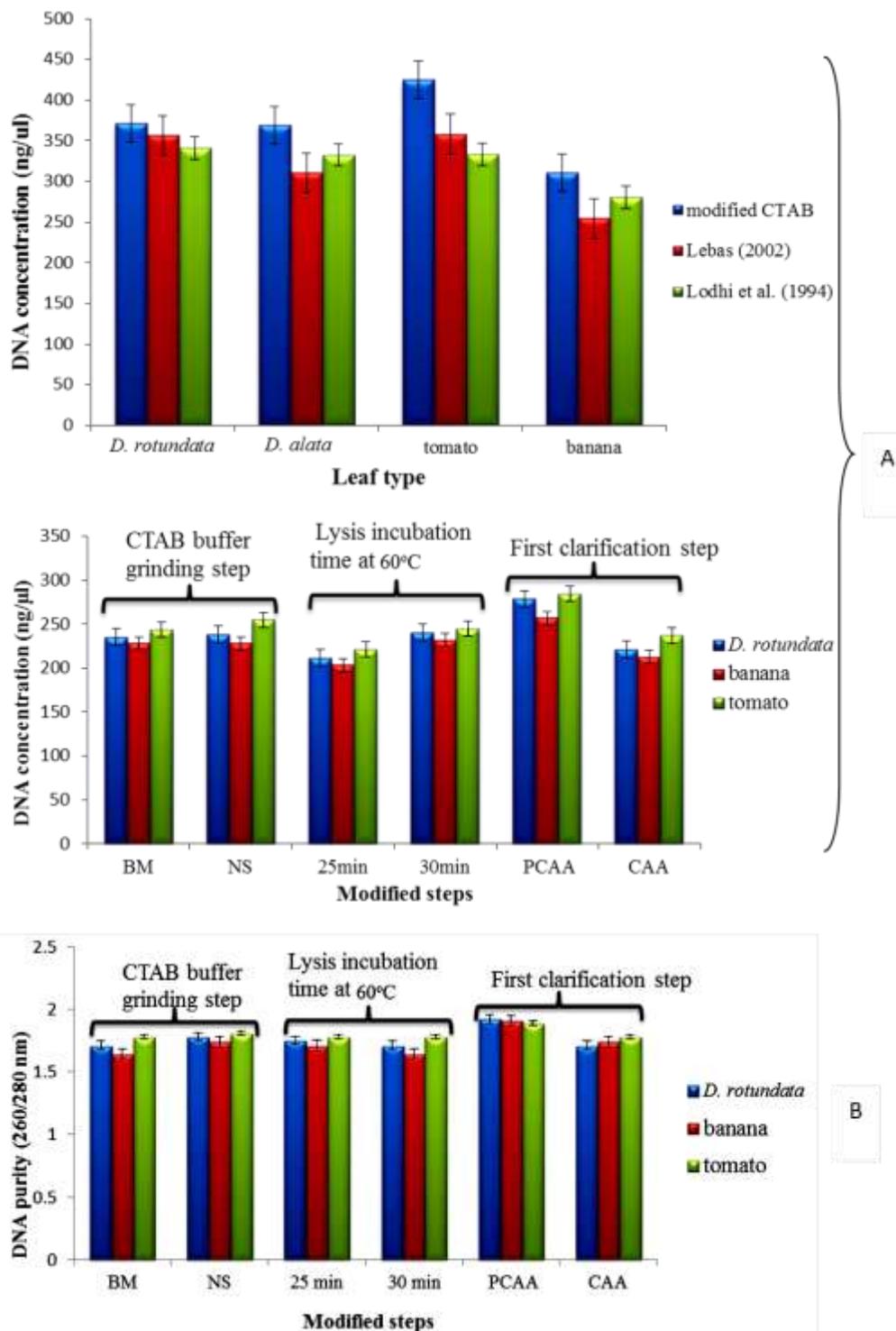


Figure 1. (A) Comparison of DNA concentration (mean \pm SEM) of Lodhi et al. (1994) method and modifications steps of Lodhi et al. (1994) method using three different plant species (banana, *D. rotundata* and tomato). Statistically (using the *t*-test) the null hypothesis is rejected as $P < 0.05$ between the DNA concentration of modified method and those of Lodhi et al. (1994) method at the modification step of 25 min and 30 min and PCAA and CAA, accepted ($P > 0.05$) for NS and BM. (B) Comparison of DNA purity (mean \pm SEM) of Lodhi et al. (1994) method and modifications steps of Lodhi et al. (1994) method using three different plant species (banana, *D. rotundata* and tomato). Statistically (using the *t*-test) the null hypothesis is accepted ($P > 0.05$) between the DNA purity of modified method and those of Lodhi et al. (1994) method at the modification step of 25 and 30 min and NS and BM rejected ($P < 0.05$) for PCAA and CAA.

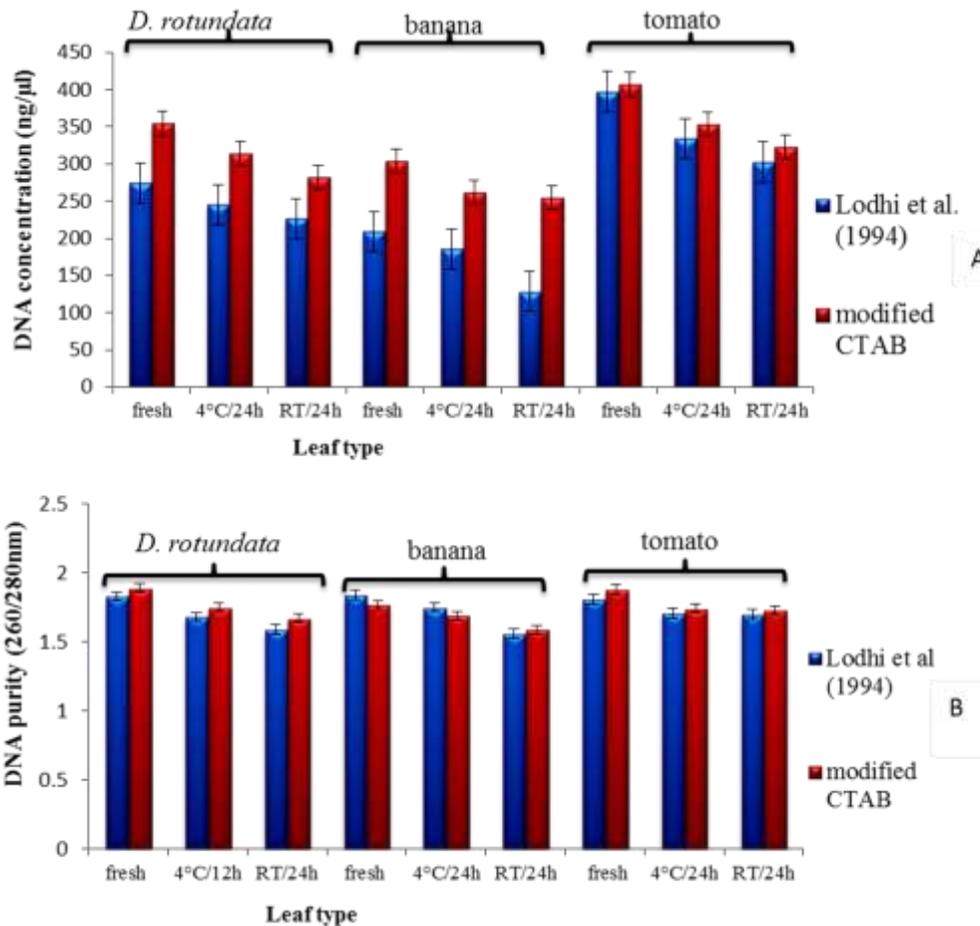


Figure 2. (A) Comparison of DNA concentration (mean \pm SEM) of Lodhi et al. (1994) and modified CTAB method on three different plant species (banana, *D. rotundata* and tomato) at three different storage conditions. Statistically (using the ANOVA test) there is a significant difference ($P < 0.05$) between the DNA concentrations of fresh and etiolated leaf samples of *D. rotundata* and banana, for tomato no significant difference ($P > 0.05$) was found. **(B)** Comparison of DNA purity (mean \pm SEM) of Lodhi et al. (1994) and modified CTAB method on three different plant species (banana, *D. rotundata* and tomato) at three different storage conditions. Statistically (using the ANOVA test) there is a significant difference ($P < 0.05$) between the DNA purity of fresh and etiolated leaf samples of *D. rotundata* and banana, for tomato no significant difference ($P > 0.05$) between the methods.

compared to the 100% success in diluted samples, indicating that dilution is vital to generate meaningful PCR results, presumably as it dilutes the concentration of PCR inhibitory substances (Figure 5).

After the method modification, a total of 231 DNAs were extracted from yam leaf samples obtained from IITA, and the DNAs were screened for the presence of badnavirus sequences by the PCR method using degenerate primers designed by Yang et al. (2003). The samples containing a band of the expected size (~579 bp) after agarose electrophoresis were scored as positive. The percentage PCR successes were evaluated (Figure 5) and this justified the effectiveness of the modified method in extracting sufficiently pure DNA. The method was also found to be suitable for extracting total DNA from dried, fresh or etiolated yam leaf tissue (Figure 5).

Purification of CTAB extracted DNA for max preps

Despite the optimized CTAB method able to extract high quality

DNA from 100 mg leaf tissue, however, this modified method failed to yield DNA of sufficient quality on the 'maxi'-preps (~3 g of leaf tissue) needed for further analysis such as Southern blot hybridisation. Therefore, further optimisation of the CTAB method had to be carried out. Yields from two additional steps were compared to the modified CTAB method. The two additional steps tried were the inclusion of a polysaccharide pre-treatment step using HEPES, and a Qiagen Tip100/G column purification of the DNA generated with the modified CTAB method. Figure 6 shows that Qiagen Tip100/G column purification of extracted DNA (QC1-QC4) yielded DNA of better quality compared to the modified CTAB method (CT1-CT4) developed and the modified CTAB method on HEPES treated ground leaf (HP1-HP4). From the result in Figure 6, it can be seen that the low molecular weight DNA/RNAs were successfully removed from the DNAs of *D. rotundata* samples (lane QC1-2) using Qiagen Tip100/G column purification. In contrast, the low molecular weight DNA/RNA was not completely removed from *D. alata* sample (lane QC3-4). This could be due to large quantities of mucilaginous substances that were observed to drop through the

Table 1. Comparison of DNA quantity and purity extracted using three different CTAB DNA extraction methods.

Plant species	DNA concentration (ng/ul) obtained using method			DNA purity (A_{260}/A_{280nm}) obtained using method		
	Modified CTAB	Lebas (2002)	Lodhi et al. (1994)	Modified CTAB	Lebas (2002)	Lodhi et al. (1994)
<i>D. alata</i>	369.08 ±2.05	310.68 ±2.12	332.52 ±2.05	2.10 ±0.05	2.07 ±0.07	1.98 ±0.02
<i>D. rotundata</i>	424.95 ±1.85	358.12 ±1.95	333.02±1.95	2.10 ±0.07	1.86 ±0.01	1.97 ±0.06
Banana	287.40 ±2.23	272.50 ±2.97	285.80 ±3.12	2.15 ±0.01	2.07 ±0.07	2.13 ±0.09
Tomato	371.10 ±2.15	356.60 ±2.45	340.70 ±1.60	2.19 ±0.04	2.11 ±0.02	2.14 ±0.08

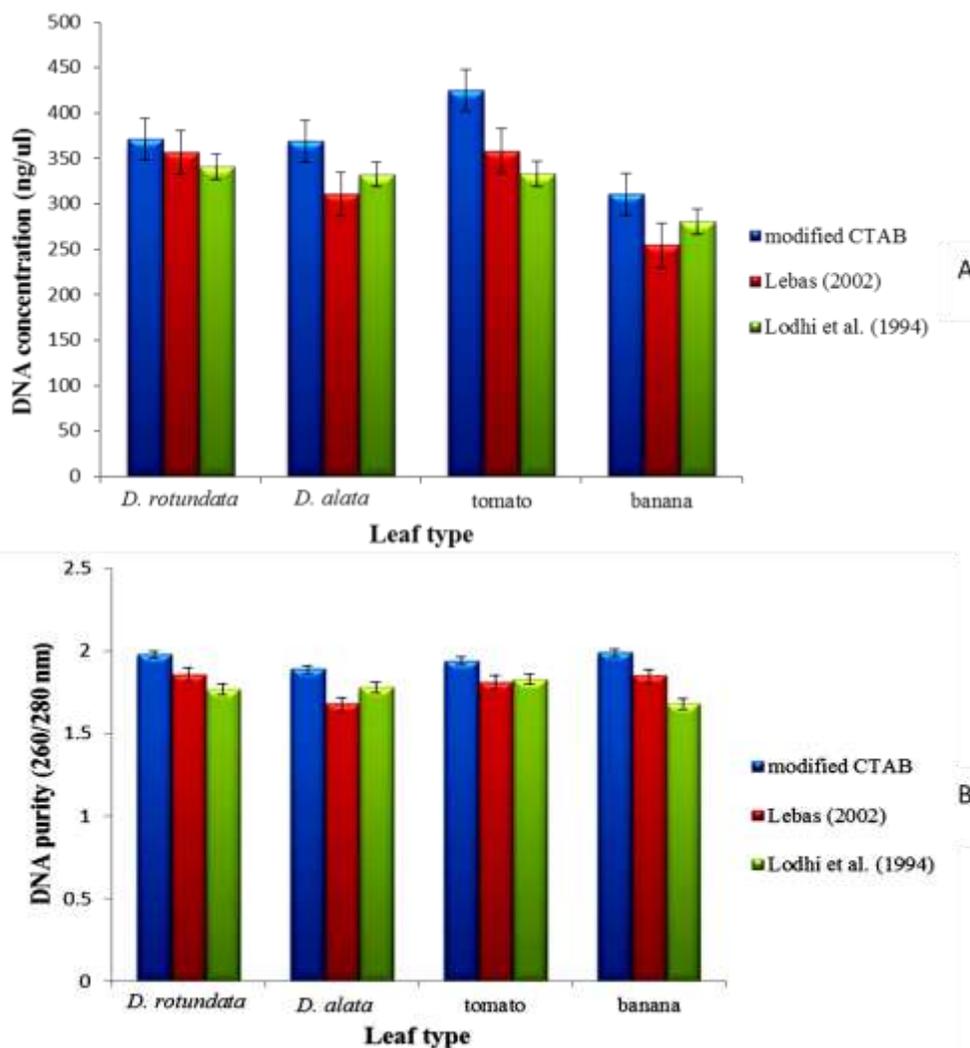


Figure 3. (A) Comparison of DNA concentration (mean ± SEM) of three CTAB methods of DNA extraction: the modified CTAB method, the Lodhi et al. (1994) method and the Lebas (2002) method using four different plant species. Statistically (using the ANOVA test), there was a significant difference ($P < 0.05$) between the results obtained with the three different methods for yam *D. rotundata* ($P = 0.0055$), *D. alata* ($P = 0.0391$) and banana ($P = 0.00672$) no significant difference ($P > 0.05$) for tomato ($P = 0.1819$). (B) Comparison of DNA purity (mean ± SEM) of three CTAB methods of DNA extraction namely the modified CTAB method, the Lodhi et al. (1994) method and the Lebas (2002) method using four different plant species. Statistically (using the ANOVA test), there is a significant difference ($P < 0.05$) between the results obtained with the three different methods for yam *D. rotundata* ($P = 0.0021$), *D. alata* ($P = 0.0035$) and banana ($P = 0.0127$) there is no significant difference ($P > 0.05$) for tomato ($P = 0.0701$).

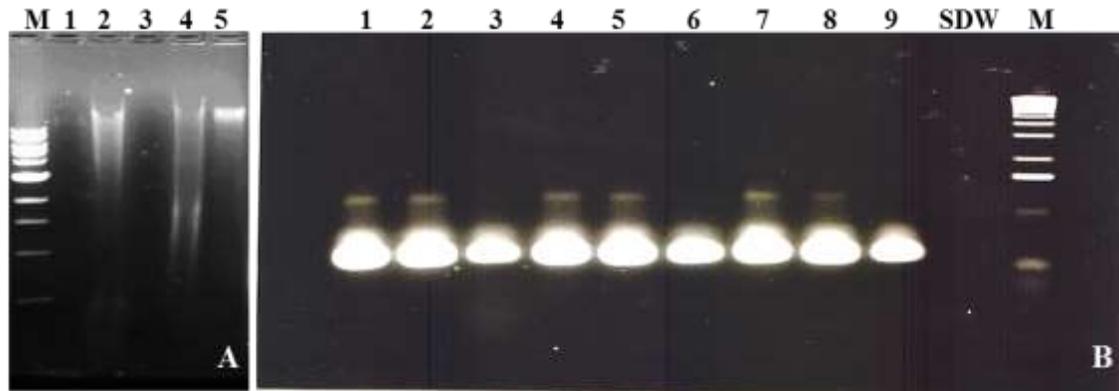


Figure 4. (A) Electrophoresis of digested and undigested extracted DNA from a *D. rotundata* leaf sample by the modified CTAB method. Lane M = 1 kb DNA ladder (NEB, UK), lane 1 = negative control (SDW as template), lane 2 = DNA digested with *EcoRI*, lane 3 = negative control (SDW as template), lane 4 = DNA digested with *TaqI* and lane 5 = undigested DNA. (B) PCR on known yam badnavirus PCR-positive samples using three different dilutions. Lane 1 = dried leaf diluted 1:1000, lane 2 = dried leaf diluted 1:100, lane 3 = dried leaf undiluted sample, lane 4 = etiolated leaf diluted 1:1000, lane 5 = etiolated leaf diluted 1:100, lane 6 = etiolated leaf undiluted sample, lane 7 = fresh leaf diluted 1:1000, lane 8 = fresh leaf diluted 1:100, lane 9 = fresh leaf undiluted sample, lane 10 = negative control (SDW as template) and lane M = 1 kb DNA ladder (NEB, UK).

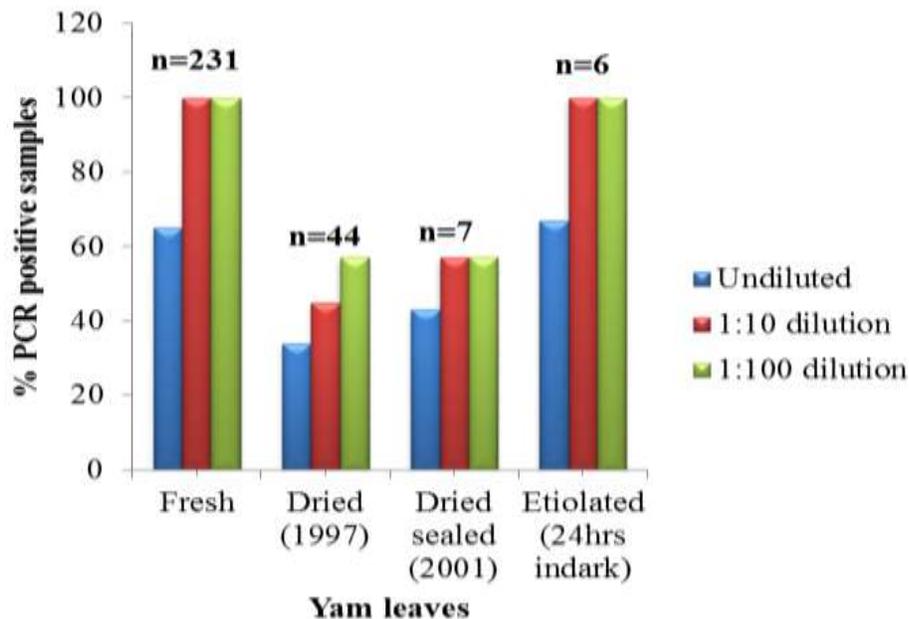


Figure 5. Percentage PCR successes of badnavirus sequences using Badna-FP and Badna-RP primers at different dilution factors for extracted DNA of *Dioscorea* species (*D. alata*, *D. bulbifera*, *D. cayenensis*, *D. dumetorum*, *D. esculenta* and *D. rotundata*) leaf samples (fresh, dried, etiolated) using the modified CTAB method.

columns for *D. alata* compared to *D. rotundata* samples during purification. This led to a slow drop of the buffers through the columns in *D. alata* samples until a gentle force was applied to enable the flow of the solutions as suggested by the manufacturers of the columns (Qiagen, UK). This additional force might have led to unwanted products passing through.

DISCUSSION

DNA extraction

Isolation of genomic DNA from yam leaves and

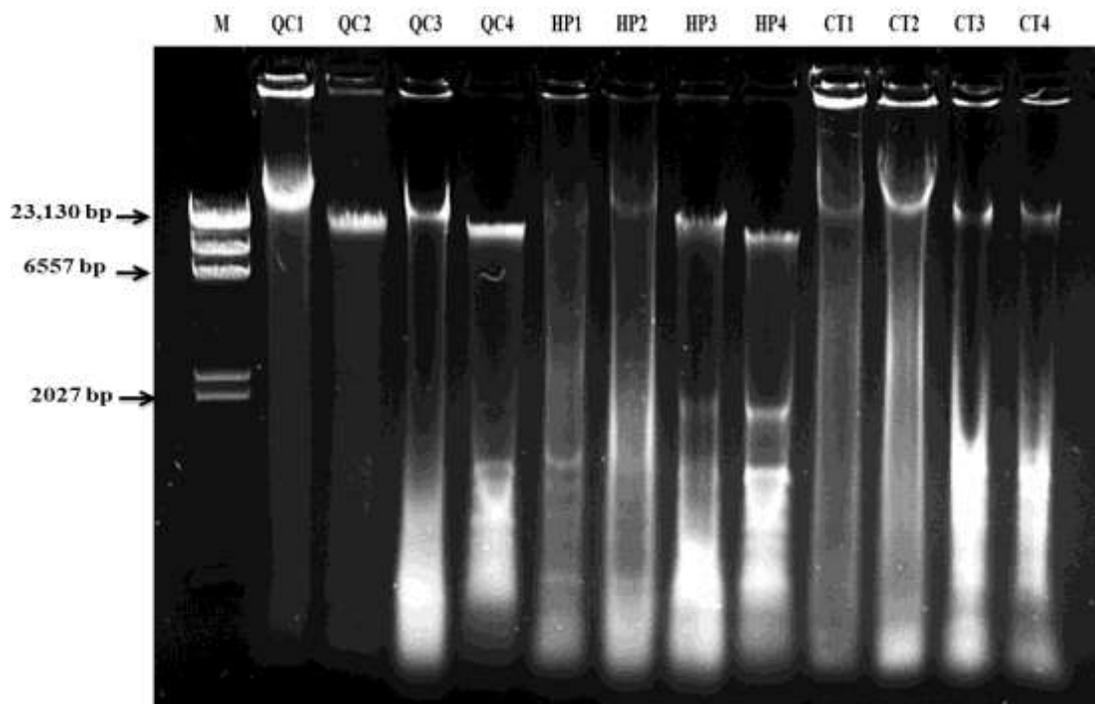


Figure 6. Total genomic yam DNAs extracted from *D. alata* and *D. rotundata* using three treatments, namely Qiagen Tip100/G column purification of extracted DNA (QC), modified CTAB method on HEPES-treated leaf (HP) and modified CTAB method (CT), all electrophoresed through a 0.8% (w/v) agarose in 0.5x TBE gel. Lane M = lambda *Hind*III ladder (NEB, UK), lane QC1, HP1 and CT1 = *D. rotundata* sample (G9), lane QC2, HP2 and CT2 = *D. rotundata* sample (G12), lane QC3, HP3 and CT3 = *D. alata* sample (G5) and lane QC4, HP4 and CT4 = *D. alata* sample (G16).

subsequent analysis such as PCR amplification, restriction digestions are complicated due to the co-purification of polyphenols and polysaccharides (Mumford and Seal, 1997). Moreover, when cells are lysed polyphenolic compounds bind to the DNA, which can cause damage to the DNA and also inhibit the activity of DNA manipulation enzymes such as polymerases or restriction enzymes. Thus, separation of these compounds from the DNA is necessary for good quality DNA extraction (Michiels et al., 2002; Sharma et al., 2008; Tiwari et al., 2012). Several methods exist for the separation of these compounds from DNA, among which is the CTAB method which was first introduced by Taylor and Powell (1982). Since then the method has been widely used for plant DNA extraction often with slight modifications (Lodhi et al., 1994; Barnwell et al., 1998; Michiels et al., 2002; Sharma et al., 2008; Abarshi et al., 2010; Attitalla, 2011; Adeyemi and Ogundipe, 2012; Tiwari et al., 2012).

In this study, the extraction method was based on the CTAB method reported by Lodhi et al. (1994). The method used was optimised by comparison of Lodhi et al. (1994), with that used by Lebas (2002). Leaf tissues used for the optimisation were banana and yam known to contain relatively high amounts of polyphenols and

polysaccharides (Sharma et al., 2008) and tomato with a lower amount of polysaccharides and high polyphenols (Peterson et al., 1997).

Lodhi et al. (1994) reported as their CTAB method was favoured over other methods based on the fact that the method had few protocol steps, was less expensive and above all the method offered DNA of good yield and quality from grapevine plant tissue that contained high amount of polyphenol and polysaccharides (Lodhi et al., 1994; Ghaffari et al., 2011). The high polyphenolic compound and polysaccharides in yam leaves were successfully removed from the extracted DNA using the optimised CTAB method, through the addition of increased concentration of PVP-40, sodium sulphite and subsequent precipitation with sodium chloride. The concentration of PVP-40 used in the modified CTAB method was similar to that used by Fang et al. (1992) and Shankar et al. (2011). The DNA values ratio of $A_{260}/A_{230\text{ nm}}$ obtained were ≥ 1.8 indicating that the extracted DNAs contained low polysaccharides (Fang et al., 1992). When the DNAs give ratios of $A_{260}/A_{230\text{ nm}} \leq 1.6$, this indicates the presence of high levels of polysaccharides in the DNA

(<http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf>).

Although, there was a difference between the mean values obtained in the purity and quantity of DNA obtained between the use of PVP-40 or β -mercaptoethanol in removing polyphenols (Figure 1A and B), these were not significant as $P > 0.05$. However, PVP-40 was chosen because it is less toxic and hence easier to dispose of its waste compared to β -mercaptoethanol. An additional advantage to the method is that samples can be processed at this initial extraction stage on a clean bench top without the need for a fume hood compared to the methods that use β -mercaptoethanol.

Another step that was optimised in the protocol was the cell lysis incubation time for which the optimum condition in this study was 60°C for 30 min. One critical step found in achieving good quality DNA from yam leaves was minimizing the time taken from grinding step to this cell lysis incubation step. The increased amounts of PVP-40 together with sodium sulphite in the CTAB buffer and increased speed of grinding to the incubation stage helped in reducing the brownish colouring of the DNA extracted.

Incorporation of a PCAA step not only yielded DNA of good purity as reported by several authors (Porebski et al., 1997; Barnwell et al., 1998; Padmalatha and Prasad, 2006), it also increased mean values of DNA yields (Figure 1A). The result highlighted the efficiency of PCAA in deproteinization compared to CAA. Two steps of washing the pellet with 70% ethanol were found to provide an increase in DNA purity in the range of 1.89 ± 0.04 to 1.92 ± 0.02 compared to the range of 1.71 ± 0.03 to 1.78 ± 0.05 obtained, when CAA was used (Figure 1B). Pure DNA preparations have expected $A_{260}/A_{280 \text{ nm}}$ ratios ≥ 1.8 (William et al., 1997). Ratios of $A_{260}/A_{280 \text{ nm}}$ below 1.6 indicate that the DNA contains large amount of proteins (<http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf>).

Although spectrophotometric method is less accurate for estimation of quantity and purity of DNA mix with proteins, nucleotide and primers because the method cannot differentiate between the contaminants and the DNA. This leads to false overestimate of DNA concentration (Sambrook et al., 1989). However, it is still the simplest and fastest method of determining DNA concentration and purity (Teare et al., 1997). However, from the results, it can be seen that the modification steps were helpful in obtaining DNA of reasonable good yield and quality.

Results from DNA concentration obtained from the comparison of three methods (Figure 3A) reveal a statistically significant difference ($P < 0.05$) among yields obtained from yam and banana leaves. However, there is no significant difference ($P > 0.05$) among the results found from tomato DNAs. This could be attributed to the lower polysaccharide and protein concentrations in the tomato leaf tissue. This indicated that both methods are suitable for extraction of DNA from the tomato leaf.

The efficiency of the modification was tested by comparing the mean concentrations of the extracted DNAs from samples stored at different conditions. The results obtained (Figure 2A and B) showed that storage has an effect on DNA quantity and purity. The lower ratio values obtained for leaves stored at 4°C and in dark at RT from the results (Figure 2A to B) indicate the presence of contaminants such as proteins and aromatic ring structure compounds in the DNA mixture that absorbed at λ 280 nm; it appears that such compounds might have been generated under these storage conditions further validation of this is necessary.

The adequate quality of the DNAs was verified by electrophoresing the extracted DNAs alongside digested DNAs. DNA migration and PCR results recorded (Figure 4A and B) indicated that the extracted DNA samples contained fewer inhibitors that interfered with restriction reaction and PCR.

PCR amplification results were obtained using different dilutions of known badnavirus PCR-positive samples of fresh, etiolated and dried yam leaf. The increased band intensity observed from the diluted samples compared to undiluted could be due to PCR inhibitors present in the undiluted samples (Figure 4B). Upon further dilution, the effect of the inhibitors was reduced. A reduced PCR success rate was recorded on dried undiluted 34% (15/44) compared to the fresh undiluted sample 69% (159/231) (Figure 5).

Cost-per-sample analysis revealed that the use of modified CTAB method for DNA extraction is cost efficient compared to the use of the Qiagen DNeasy plant kit. The lower cost of extracting DNA with the modified CTAB method could be of advantage over the kit method considering that yams are produced in developing countries where resources to purchase reagents and kits are more restricted, yet labour is relatively inexpensive. However, the cost saving should only be realised if the quality of the DNA is better and downstream applications are not affected as the cost of PCR, cloning, sequencing and restriction digestion of the extracted DNAs far outweigh the cost of DNA extraction. Furthermore, considering the poor waste management systems in many sub-Sahara African laboratories, the disposal of PCAA from the modified CTAB method will pose a problem and therefore in these situations the Qiagen DNeasy plant kit method could be favoured over the modified CTAB method.

For laboratories with adequate waste disposal systems, an additional advantage of the modified CTAB method over the kit method is that it can be performed at any desired scale, whereas the kit method can only be performed at the scales supplied by the kit manufacturer due to the DNA-binding capacity of the columns supplied. Although a comparison of the success rate of PCR screening between the optimised CTAB method and the kit method was not undertaken in this study, similar modifications of a CTAB method have shown efficient

PCR amplification of DNAs from yam samples compared to the Qiagen DNeasy plant kit extraction method (Lebas, 2002).

Purification of CTAB extracted DNA

Separation of polysaccharide from DNA is one of the major challenges of DNA extraction from plants material containing high levels of polysaccharide (Sharma et al., 2002; Ghaffari et al., 2011). Separation of these two polymers is usually achieved by the high salt concentrations used in the extraction buffers and the DNA precipitation step (Fang et al., 1992). Evidence arising from this study indicates that the modified CTAB extraction yielded good quality DNA for a 'mini prep'. The method could not yield sufficient DNA of high quality for a 'maxi prep'. To obtain higher quality DNA of sufficient quantity DNA from 'max prep' further purification of the 'maxi prep' extracted DNAs had to be carried out. The purification of extracted DNAs using a Qiagen Tip100\G column was found to yield DNA of much better quality compared to adding a HEPES-treatment to leaves (Figure 6), a suggestion made by IITA to overcome this problem (R. Bhattacharjee and G. Gezagegh, personal communication). However, the problem experienced with incorporating a Qiagen Tip100\G columns purification step was that about two-thirds of the DNA was lost during purification.

PCR detection of yam badnavirus sequences

In this study, the PCR-based diagnostic assay used for the detection of badnavirus sequences was similar to those used successfully for detection of many badnavirus sequences in different tropical food crops such as banana (Geering et al., 2000), cacao (Muller et al., 2001), pineapple (Thomson et al., 1996), sugarcane (Braithwaite et al., 1995), taro (Yang et al., 2003) and yam (Eni et al., 2008; Kenyon et al., 2008; Lima et al., 2013). All the PCR tested samples collected from IITA were badnavirus positive. This was in agreement with the Seal et al. (2014) that all West African *D. rotundata* samples are badnavirus positive. Dilution of extracted yam DNAs before PCR was critical as this was found to increase the success of PCR amplification (Figure 5).

The 100% PCR-positives obtained from diluted DNAs of the 151 West African native yams (*D. cayenensis* to *D. rotundata* complex) was not surprising considering that earlier studies also reported 100% PCR-positives from *D. cayenensis* to *D. rotundata* samples from West Africa (Seal et al., 2014). The latter and Kenyon et al. (2008) also reported PCR-positives from ELISA-negative samples. Therefore, these results suggest that some of the PCR-positives might originate from EPRVs. Furthermore, this also reflect the high incidence of badnavirus infection rates as reported from several West

African field surveys, recording up to 100% badnavirus incidences (Eni et al., 2008; Asala et al., 2012; Yeyeh et al., 2014; Kumar, unpublished).

Several factors are known to affect the efficiency of badnavirus detection by PCR, among which is the choice of primers. A high degeneracy of primers may lead to non-specific amplification of targeted DNA, which will result in the generation of non-specific PCR products (Linhart and Shamir, 2005). Lebas (2002) reported that non-specific PCR products were obtained using degenerate primers designed from BSV (Lockhart and Olszewski, 1993) on South Pacific badnavirus isolates infecting yam. However, degenerate primers (Badna-FP and Badna-RP) used for this study were designed by Yang et al. (2003), and were used successfully for the detection of badnaviruses from yam breeding lines and landraces collected from IITA. The primers have also been used for the amplifications of DBSNV (Seal and Muller, 2007), detection of yam-infecting badnaviruses from the South Pacific (Lebas, 2002) and West Africa (Eni et al., 2009).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to thank Professor Susan E. Seal, professor in Molecular Biology at the Department of Agriculture, Health and Environment Natural Resources Institute University of Greenwich UK for her guidance.

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