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AN IMPROVED METHOD FOR INHIBITOR FREE NUCLEIC ACIDS FROM POLYPHENOL RICH LEAVES OF *MUSA SPP*

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ABSTRACT

Excessive presence of polysaccharides, polyphenol and secondary metabolites in banana plant affects the quality of DNA and it leads to difficult in isolating good quality of DNA. An optimized modified CTAB protocol for the isolation of high quality and quantity of DNA obtained from banana leaf tissues has been developed. In this protocol a slight increased salt (NaCl) concentration (2.0M) was used in the extraction buffer. Polyvinylpyrrolidone (PVP) and Octanol were used for the removal of polyphenols and polymerase chain reaction (PCR) inhibitors. Proteins like various enzymes were degraded by Proteinase K and removed by centrifugation from plant extract during the isolation process resulting in pure genomic DNA, ready to use in downstream applications including PCR, quantitative polymerase chain reaction (qPCR), ligation, restriction and sequencing. This protocol yielded a high molecular weight DNA isolated from polyphenols rich leaves of *Musa spp* which was free from contamination and colour. The average yields of total DNA from leaf ranged from 917.4 to 1860.9 ng/ìL. This modified CTAB protocol reported here is less time consuming 4-5h, reproducible and can be used for a broad spectrum of plant species which have polyphenol and polysaccharide compounds.

Figures : 06 References : 20 Tables : 02
KEY WORDS : CTAB, DNA extraction, *Musa spp*, Octanol, Proteinase K, PVP.

Abbreviations

CTAB : Cetyl trimethyl ammonium bromide
TE buffer : Tris EDTA Buffer
EDTA : Ethylene diamine tetra acetic acid
PVP : Polyvinyl pyrrolidone
ISSR : Inter Simple Sequence Repeat

Introduction

Banana (*Musa spp* L.;*Musacease*), commonly known as the 'Apple of Paradise' is a popular tropical fruit, especially in Asia[12]. In conjunction with other biotechnological applications, such as tissue culture and somatic embryogenesis, the use of high quality genomic DNA would advance molecular studies on this tropical species[14]. The isolation of high-quality DNA is important in any molecular biological work because contaminants such as proteins, polyphenols and polysaccharides may interfere with enzymes such as restriction enzymes (in blotting techniques) and Taq polymerase (in polymerase

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chain reaction (PCR))[1]. Isolation of high quality nucleic acids from plant tissues rich in polysaccharides and polyphenols is often a difficult task. The presence of these substances can affect the quality and/or quantity of the nucleic acids isolated[8]. Numerous protocols have been published for the isolation of total DNA from different plant tissues; the majority are not completely satisfying as they may be time consuming, technically complex, require more centrifugation steps and are specific to a particular plant species[7,11,13]. Commercially available DNA isolation kits provide high throughput and reduced labor time, though this is not always true for all plant materials. Additionally, their availability and high cost can be limiting in certain developing countries, especially when handing a large number of samples and considering experiments with limited financial resources[2,15]. Since the biochemical composition of plant tissues vary with the species, DNA isolation protocols need to be optimized for each[20]. Though the basic idea behind the DNA extraction is not very complicated, growing number of DNA isolation protocols for specific plant species suggest that the extraction procedures are not always simple and published protocols are not necessarily reproducible for all species[16]. This necessitates attempting various reported methods and to bring necessary modifications so that a suitable protocol may be arrived. High percentage of polyphenols and other secondary metabolites in banana make the DNA isolation difficult. These plants are reported to have higher level of polyphenols and tannin content[18]. These phenols are inhibiting *Taq* polymerase during PCR amplification[17].

In all the studies of molecular biology, isolation of a good quality DNA is an important pre-requisite. Banana leaves were found to contain high polyphenolic contents and polysaccharides which made the DNA extraction difficult task. The present investigation was therefore, undertaken to test the suitability of available methods for DNA isolation and make modifications, wherever necessary, to get genomic DNA of good quality and yield for use in further studies. The principle modification in this method was increased in high salt concentration (2.0M) in the extraction buffer was used. In addition, Polyvinylpyrrolidone (PVP), Octanol and Proteinase K were included as an optional step for phenol rich leaves of banana. This compound breaks the bonds between nucleic acids and phenolics, preventing loss of DNA and increasing DNA yield. These modifications produced consistently pure and high quality DNA suitable for further molecular analysis.

Material and Methods

Plant material

For the conducting of this experiment, cigar leaves from different cultivars like Rasthali (AAB), Grand Naine (AAA), Hill banana (AAB), Nendren (AAB) and Ney pooven (AB) were collected from the experimental field of the Department of Fruit Crops, Faculty of Horticulture, Tamil Nadu Agriculture University, Coimbatore, India. Samples collected from the donor plants were quickly wrapped in aluminium foils, transported to the laboratory using liquid nitrogen contain box and stored at - 80°C till being used for the extraction of DNA.

DNA extraction

The leaves were subjected to three genomic DNA extraction protocols, which comprised Traditional CTAB[5] (Method-I), Modified CTAB (Method-II) and Dellaporta[4] (Method III). The method-II has been discussed.

DNA isolation (Method II)

Leaf samples of about 0.5 g were weighed and transferred to a pre-chilled mortar and pestle. The powder was transferred to a centrifuge tube and 10 mL of pre-warmed (65° C) CTAB buffer (2% cetyl trimethyl ammonium bromide, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2 M NaCl, 40 ìL Proteinase K (20 mg/ml), 1% (v/v) â -mercapto ethanol and 3% PVP) were added to each sample and tubes were incubated at 65°C for 30 min with shaking for every 10 min. After incubation, samples were kept in ice for 30 min. The samples were then centrifuged in a bench top centrifuge at 12000 rpm for 10 min (4°C). An equal volume of Phenol: chloroform: octanol (25:24:1) v/v was added, mixed well by gentle inversions for 20 minutes and then centrifuged at 12000 rpm for 10 minutes (4°C). The supernatant was carefully decanted and transferred to a new tube and DNA was precipitated by adding of pre chilled isopropanol, mixed gently inversion incubated at -80°C for 1 hr followed by 10 min of centrifugation. Dissolved DNA was transferred to microfuge tube containg 100 ìL of sodium acetate and 400 ìL of 100 % ice cold ethanol. The tubes were mixed gently and incubated at -20 °C for 20 min before centrifugation. The DNA pellet was washed using ice-cold 70% ethanol by briefly vortexing and the tubes were again centrifuged at

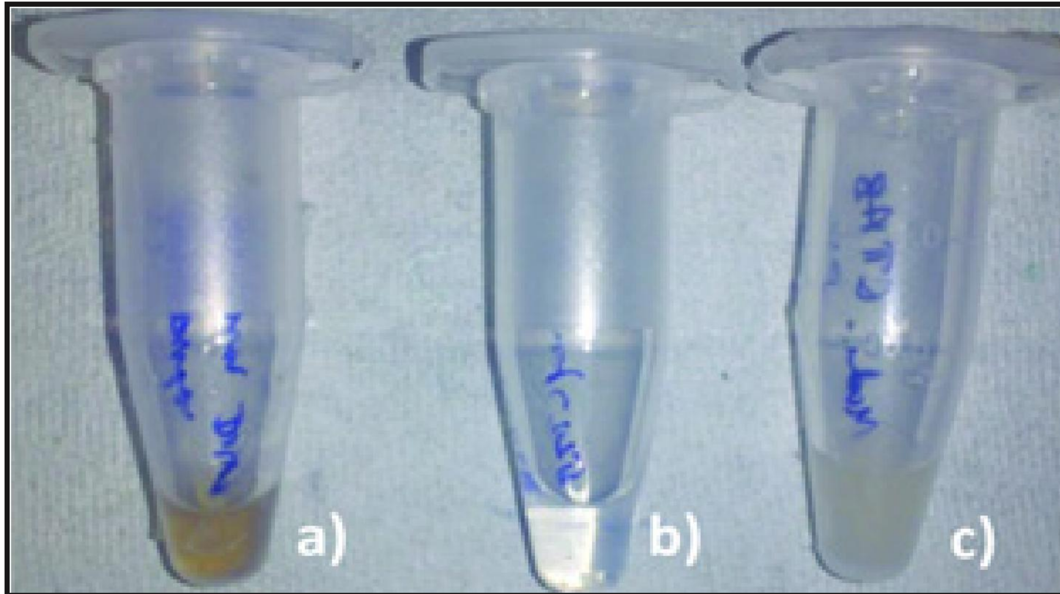

Fig.1 : Genomic DNA extractions from *Musa spp*. (a) Traditional CTAB-based method (b) Modified CTAB (c) Dellaporta method

10000 rpm for 2 min. The ethanol was removed using a pipette and the pellets dried under vacuum for 10 min before resuspending in 100 ìL of nuclease-free water or 1X TE buffer (1mM Tris + 0.1mM EDTA, pH 8.0). The total nucleic acids extracted were treated with 10 mg/ml of RNase A by incubating with 1 µl of RNase A with 100 µl of total nucleic acids at 37ÚC for 1h. The RNase A reaction was terminated by incubating the mixture at 65ÚC for 10 minutes. The nucleic acids were stored at -20ÚC.

DNA quantification

The yield of DNA per 0.5 g of leaf sample extracted was measured by using Nanodrop spectrophotometer ND-1000. The purity of DNA was determined by calculating the ratio of absorbance at 260/280 and 260/230 nm. DNA concentration and purity was further tested by running the samples on 0.8% agarose gel. The suitability of isolated DNA for molecular analysis was further determined by digesting the genomic DNA with *Kpn* I and *Hind* III restriction endonucleases. The reaction mixture was carried out in 20 µl volumes containing 4 µl of DNA, 2 µl of 10X assay buffer, 2 µl of BSA (10 mg/ml) and 1 µl of restriction enzymes at 37 ºC overnight. The

TABLE-1: List of primers used to amplify DNA

Primer name	Sequence (5' - 3')	Origin of primers
USB 811	$(GA)_8C$	NCS-TCP - National Certification System for Tissue Culture Raised Plants, Department of Biotechnology, Government of India, New Delhi
USB 818	$(CA)_8CG$	
USB 836	$(AG)_8YA$	

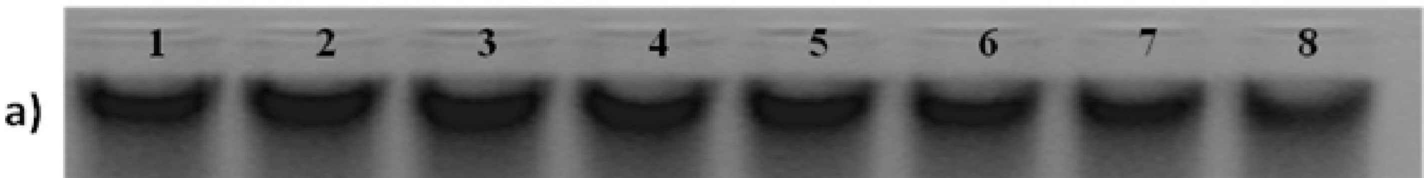

Fig. 2: Genomic DNA of banana 0.8% agarose gel electrophoresesed. a) Modified CTAB; Lane 1 to 2: Rasthali; **Lane 3 to 4**: Grand Naine; **Lane 5 to 6**: Hill banana; **Lane 7 to 8**: Nendren

TABLE-2: Comparison of quality and quantity of DNA from leaves of banana using three DNA extraction methods

Extraction methods	Cultivar	*DNA Concentration (ng/ìL)	Absorption ratio (260/280)nm	Absorption ratio (260/230)nm
CTAB	Rasthali	470.2	1.87	1.79
	Grand Naine	538.67	1.96	0.35
	Hill banana	237.67	1.99	0.98
	Ney Poovan	512.50	1.84	1.72
	Nendren	362.50	1.28	0.93
Modified CTAB	Rasthali	1722.4	1.92	1.52
	Grand Naine	1860.9	1.72	1.89
	Hill banana	917.4	1.90	1.69
	NeyPoovan	1147.9	1.81	1.71
	Nendren	1289.9	1.75	1.60
Dellaporta	Rasthali	192.20	1.84	1.15
	Grand Naine	181.83	1.72	1.46
	Hill banana	122.82	1.84	0.52
	NeyPoovan	141.97	1.35	0.45
	Nendren	127.90	1.23	1.83

*Concentration estimated using NanoDrop Spectrophotometer ND-1000

digested DNA was electrophoresed on 1.0 % agarose gel and visualized using Syngene Gene snap gel doc system

PCR amplification using Actin and ISSR primers

A polymerase chain reaction for amplifications of Actin and ISSR primer (Table-1) was carried out in a 20 µl volume for all DNA preparations. PCR reactions were carried out in a 20 ìl reaction mixture volume containing 50 ng of template DNA, 100 ìM dNTP mix, 0.6 ìM of random primers, 1X Taq DNA polymerase buffer containing 15 mM $MgCl_2$ and 1.0 U of Taq polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). PCR reaction was performed in a Thermal Cycler (Bio-Rad, C 1000™) with the following conditions: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C and a final extension step 10 min at 72°C. The same PCR condition as above was used for the amplification of the Actin gene except that the primer annealing temperature of 58ÚC was used. The amplified products were resolved by electrophoresis on a 1.0 % (w/v) agarose gels run in 1X TBE buffer and the resulting fragments were scored under UV light using a gel documentation system (Syngene).

Amplification of banana endogenous gene by RT-q PCR

To estimate the amount of endogenous Actin gene in the DNA extracts, real time PCR reactions were carried out Bio-Rad (Thermal Cycler, C 1000™). The product size was 98 bp. The real time PCR mix contained 1x iTaq™ universal mastermix (SYBR® Green supermix), 300nM each primer, 10ng of template DNA, making a final volume of 10. The primer sequence of actin was 5'GATTCTGGTGATGGTGTGAGC3'-F and 5'ATAATCCAGCGCAACGTAGG3'-R. Thereafter, all the quantifications were subjected to the following standard PCR reaction conditions: Initial hold at 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 5 s and 82°C for 5 s. A final cycle of 95°C for 15 s, 60°C for 15 s, 95°C for 15 s was performed to provide dissociation curves (melting curves) for each sample, for determination of amplification specificity.

Technical Notes

1. β-mercaptoethanol, PVP and Proteinase K should be added to the CTAB buffer just prior to use. â-mercaptoethanol and PVP inhibit the oxidation of polyphenols and Proteinase K adsorbs polyphenols, thereby preventing their interaction of DNA
2. Never shake the DNA solution violently to avoid mechanical shear of DNA
3. Drying the DNA pellet using a Vacuum concentrator plus (Do not use the Vacuum concentrator for greater than 10 min)
4. Air drying of DNA pellet (Removing the residual ethanol is critical, especially if the DNA is to be used directly for PCR)
5. TE contains a chelator that can affect reactions such as PCR, or restriction digests. DNA in TE should be suitably diluted before use in such reactions
6. If you have many samples, store the ground powder at -20ÚC temporarily after grinding with liquid nitrogen, then add buffer to all samples at the same time when grinding has been completed.

Results and Discussion

Isolation of genomic DNA, ideal for a wide range of molecular biological applications like preservation of the genetic material and biodiversity studies of *Musa* species for their suitable exploitation. At present several banana varieties have many synonyms in different regions which make identification difficult. Differentiation of cultivars through morphological features is inefficient and inaccurate. Various types of DNA based molecular techniques are used to evaluate the genetic variability of plants. Isolation of highly purified plant DNA is difficult, particularly from plants like banana with high polyphenolic compounds[3]. These approaches require both high-quality and quantity of DNA, for which *Musa* species presents a great challenge[19]. Different extraction methods significantly influenced the quality of DNA and its amplification efficiency of ISSR markers. The CTAB based method yielded translucent creamish DNA with high viscosity. In this protocol polyphenols co-precipitated with DNA on addition of isopropanol formed large sticky cream colored pellet which could not be dissolved in TE buffer and was unsuitable for electrophoresis. In Dellaporta method, used of anionic detergent SDS, also resulted in recovery of DNA contaminated with polyphenols making it opaque and difficult to dissolve. Such DNA samples stuck to the wells during electrophoretic separation. To overcome this problem a modified method incorporating LiCl was tested. However, this modification tried yielded low

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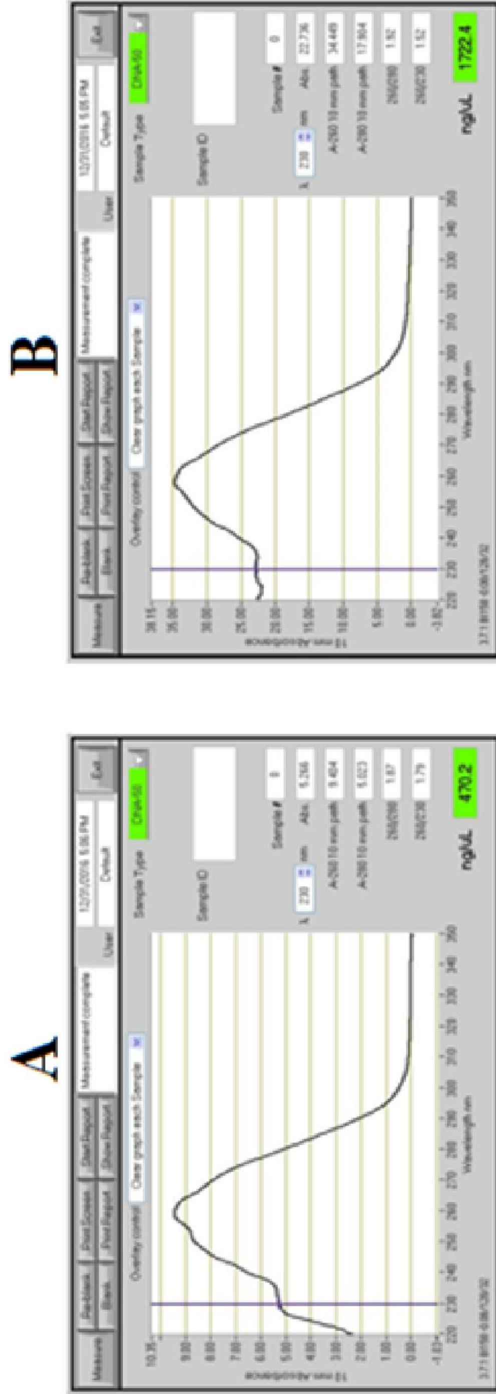

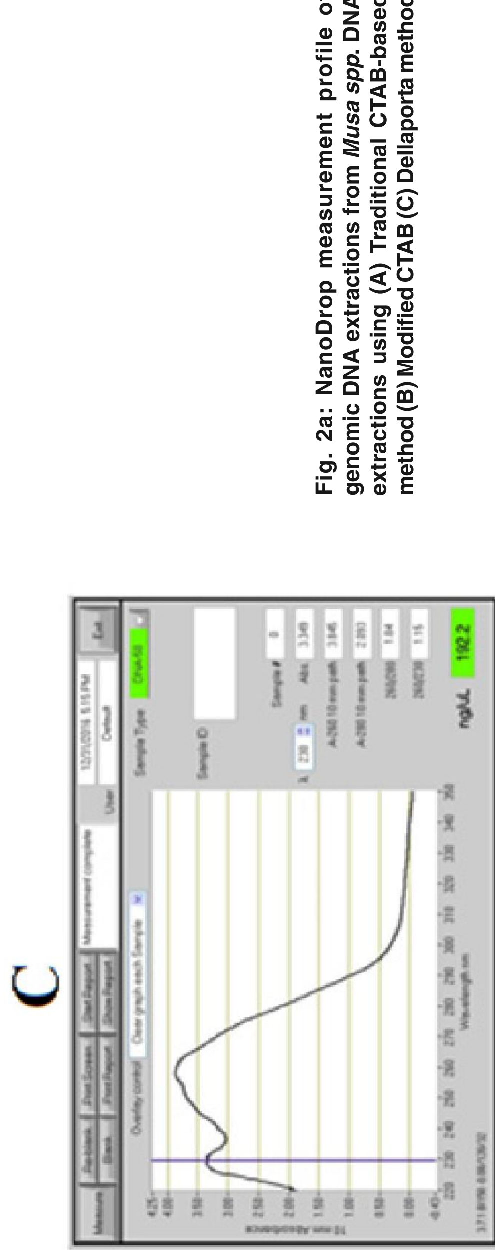

Fig. 2a: NanoDrop measurement profile of genomic DNA extractions from *Musa spp.* DNA extractions using (A) Traditional CTAB-based method (B) Modified CTAB (C) Dellaporta method

quality DNA. In marked contrast, the DNA purified by modified CTAB based method was most suitable for extraction of desired quantity and quality of genomic DNA from phenol rich leaf tissue (Fig.1). Also, the A260/280 ratio ranged from 1.87 to 2.01, indicating a low protein contamination (Table-2).

A modified CTAB method has been developed for easy isolation of inhibitor-free genomic DNA from even the toughest plant leaf samples, including those high in polyphenols and polysaccharides. To prevent the solubilisation of polysaccharides in the DNA extract, high salt (NaCl) concentration (2.0 M) in the extraction buffer was used for the precipitated DNA. High-salt buffer (1.5-2.0 M NaCl) proved effective isolation of genomic DNA from muskmelon, cucumber, potato, soybean, and geranium[6]. At this level, the polysaccharides remained in the solution and were discarded with the ethanol supernatant, decreasing the levels of polysaccharide. PVP and octanol were used for the removal of polyphenols that are known as PCR inhibitors and proteins like various enzymes were degraded by proteinase K and removed by centrifugation from plant extracts during the isolation process resulting in pure DNA is ready to be used in downstream applications including PCR, quantitative PCR, real-time PCR and sequencing. Polyphenolics occur at different concentrations in the leaves, bark and fruit of higher plants. An important characteristic of many polyphenolics is their propensity to form complexes with nucleic acids. Hence, a variety of protocols have been developed to avoid inhibition of molecular biological reactions[10]. In this research, we have included PVP and octanol in the extraction buffer, alleviating the inhibition of Taq DNA polymerase associated with unknown components, polyphenols, present in several crude DNA preparations and thus increasing the utility of our simple method. It is exceptionally good at absorbing polyphenols during DNA purification. Polyphenols are common in many plant tissues and can deactivate proteins if not removed and, therefore, inhibit many downstream reactions like PCR. The initial stage of DNA isolation is to break open the cell and release the cytoplasmic contents, which includes the nucleus, in which we find DNA. Proteinase K is a protease which is used to digest the cell surface proteins. When cell surface proteins are digested, the integrity of the cell membrane is compromised leading to cell lysis. Most protocols for the extraction of DNA from fresh tissue or cultured cells require tissue to be incubated with proteinase K for 12–24 h. An incubation time of 18 h for the proteinase K extraction technique was a very efficient procedure, capable of extracting high molecular weight DNA (more than 20 kilobases) from as little as one frozen section of the fresh tonsil[9]. In this modified CTAB method, the tissues were incubated with proteinase K for 30 min.

The principal modifications in this method included use of 1.4 M salt (NaCl) concentration instead of 2.0 M in the extraction buffer. This high salt concentration (2.0 M) allowed maximum yield (Fig.2). Another modification included supernatant with Phenol: chloroform: octanol. This increased the yield and quality of DNA because pooling of supernatant with octanol allowed binding of more DNA. CTAB method with these modifications appears to have excluded DNA contamination like polysaccharides, polyphenols and sticky substances, which otherwise hamper DNA isolation and purification (Fig.2a). This recovered DNA samples when digested with the restriction endonuclease Kpn I and Hind III, showed complete digestion (Fig.4). The isolated DNA using modified CTAB method was tested in Actin gene PCR for DNA samples selected randomly gave expected 664bp amplicons as shown in Fig. 3, confirming that the DNA was intact and of PCR quality. Estimated the overall quality of the DNA extracted from phenol rich leaves of banana using RT-qPCR. Our results suggest that a lot of variation was noticed from three different DNA extraction methods. Off these methods, intact band was observed in modified CTAB compare to traditional CTAB and Dellaporta (Fig.6). This method may come in helpful in the detection of virus, genetically modified organisms (GMO) in particular for samples that contain low level of the viral pathogen/genetically modified gene. High concentration of polyphenols in banana leaves might be interfering with PCR reactions in CTAB and Dellaporta. This result suggests that appropriate DNA dilution is critical to successful PCR, particularly when plant samples rich in polyphenols are used as source materials. The PCR products produced by ISSR analysis showed reproducible and clear scorable banding pattern (Fig.5). Thus, the protocol proved to be advantageous because of its simplicity and affordable reagents, besides achieving intact high molecular weight and purity of genomic DNA. The isolated DNA proved amenable to PCR amplification including actin and ISSR analysis. In

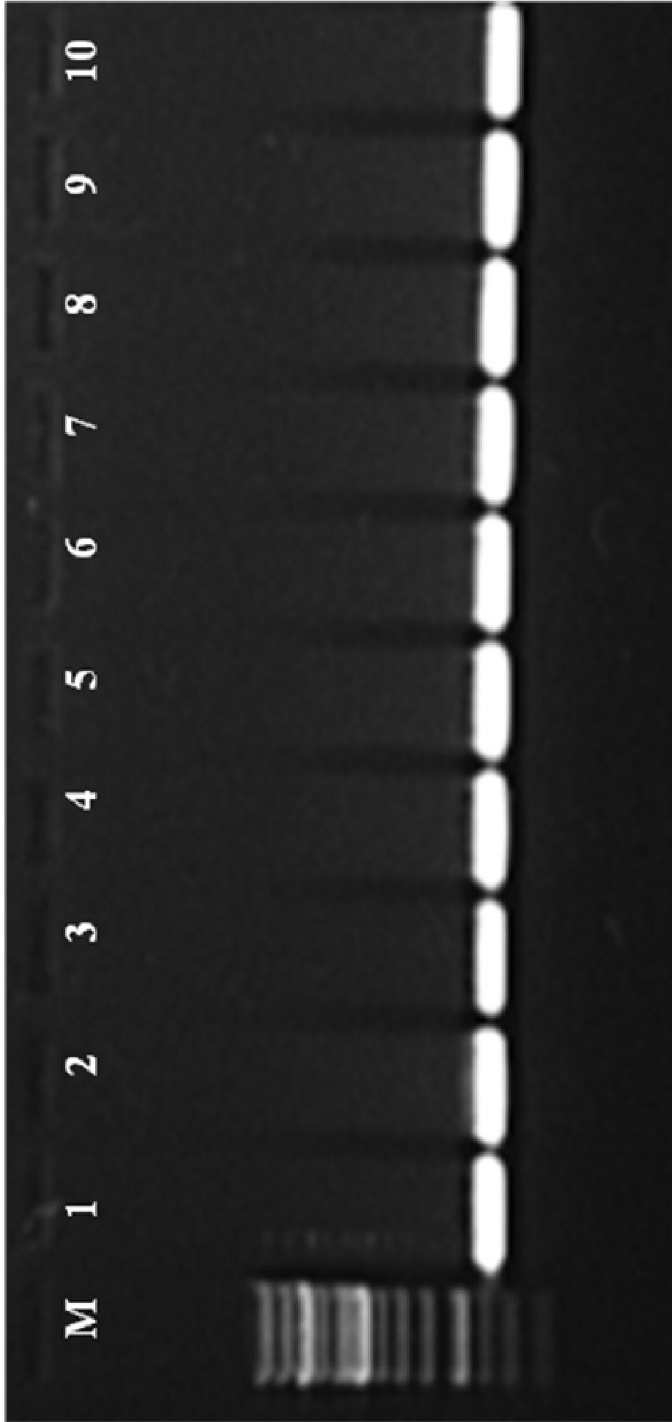

Fig. 3: PCR product resolved in 0.8% agarose gel using Actin specific primer. Lane M: 1 kb Ladder; **Lane 1 to 2**: Rasthali; **Lane 3 to 4**: Grand Naine; **Lane 5 to 6**: Hill banana; **Lane 7 to 8**: Neypoovan: **Lane 9 to 10**: Nendren. PCR product amplification size is 664 bp.

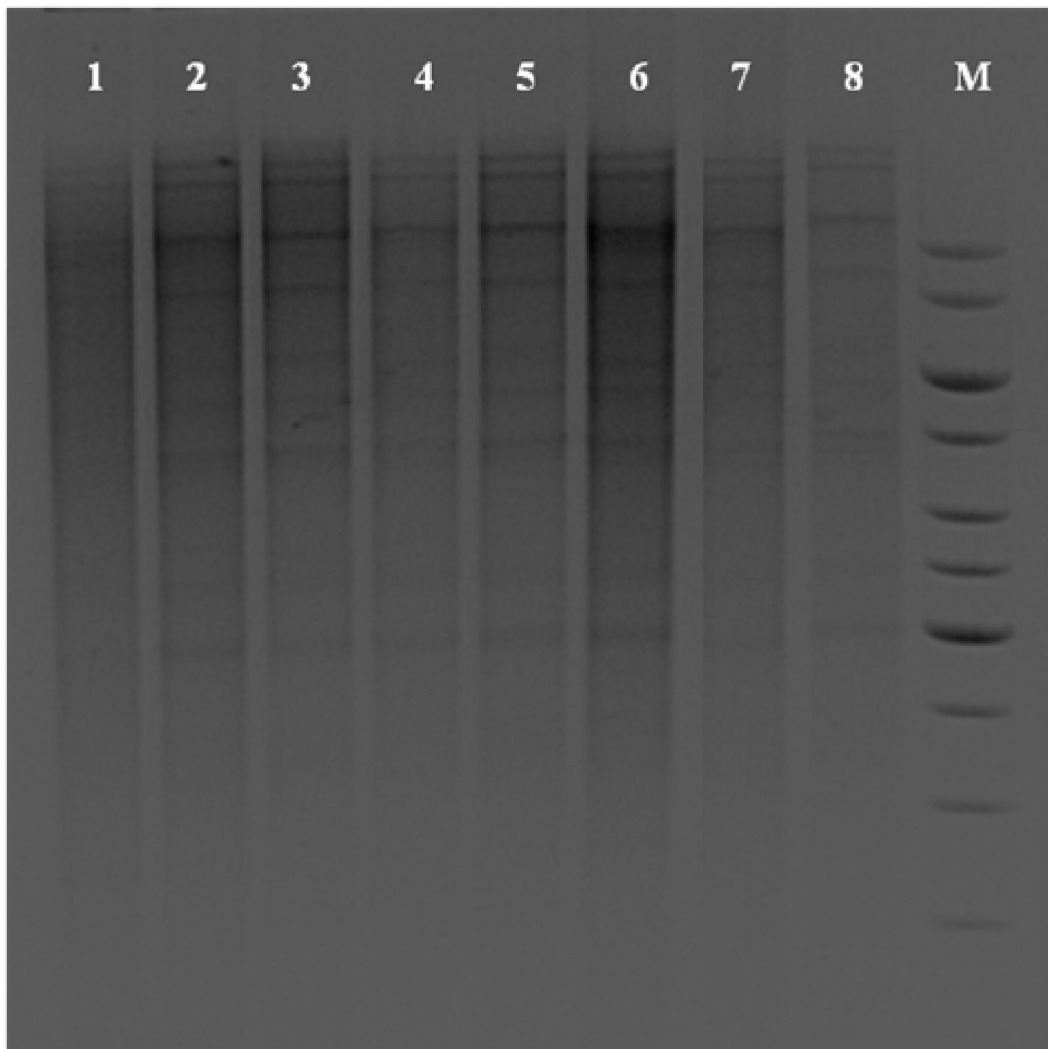

Fig. 4: Restriction digestion of DNA. Lane M: 1 kb Ladder; Lane 1 to 4: *Kpn* I; Lane 5to 8: *Hind* III.

conclusion, modified CTAB method, with higher salt concentration binds to polysaccharides and additions of PVP, octanol and proteinase K prevents the browning of DNA caused by oxidation of polypheols and increasing solubility in ethanol so that they do not co-precipitate with the DNA. This could be adopted as standard method for isolation of DNA from *Musa spp* or similar materials rich in polyphenolics and polysaccharides. This procedure explained here is fast, simple and more reliable enabling the processing of large number of samples with ease.

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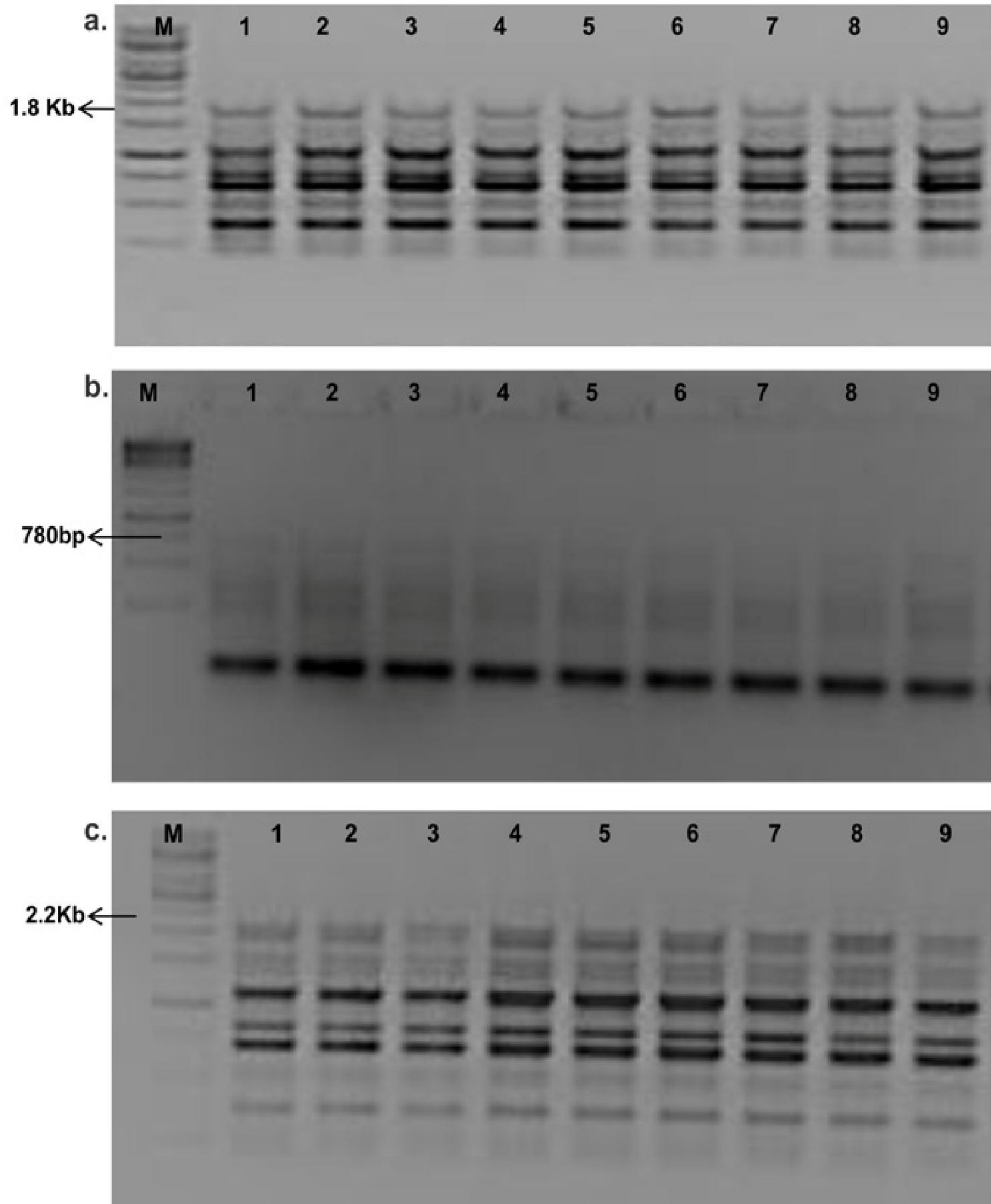

Fig. 5: PCR results of nine DNA samples were amplified by (a) primer USB 811 (b) primer USB 818 (c) primer USB 836. M is 1 Kb ladder marker

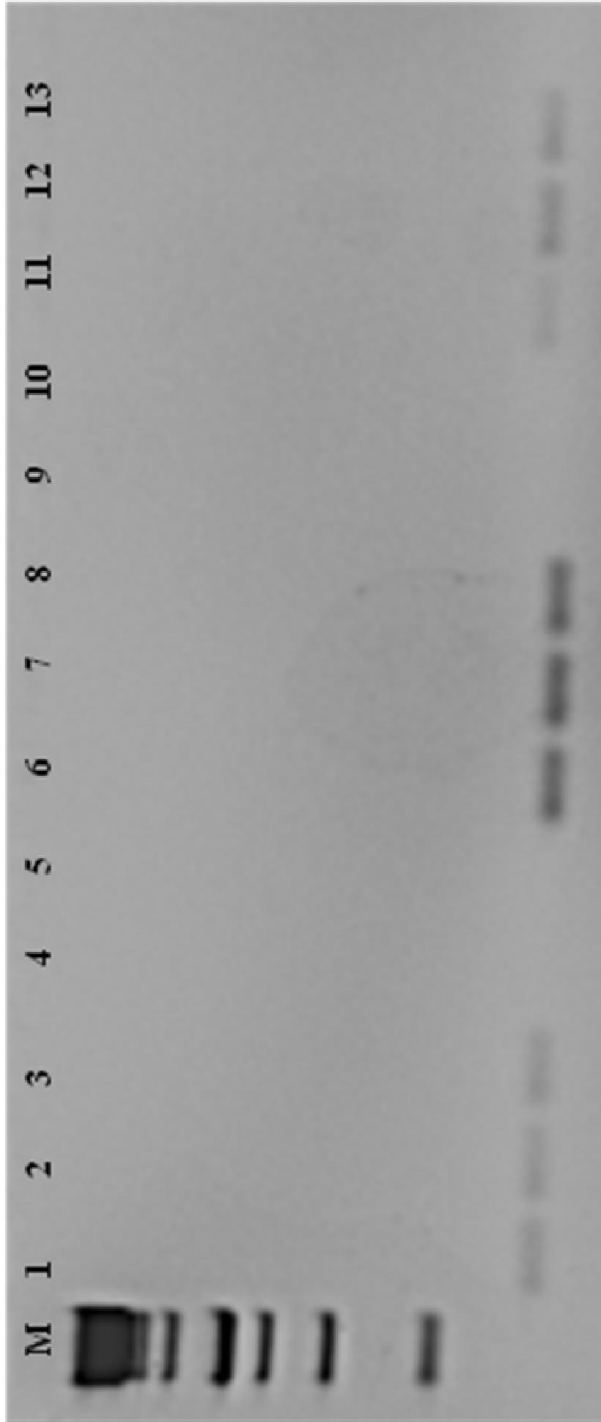

Fig.6: Agarose gel electrophoresis of Actin specific primer using RT-qPCR. Lane M: 100bP Ladder; Lane 1 to 3: Traditional CTAB; Lane 4 to 5: water control; Lane 6 to 8: Modified CTAB Lane 9 to 10: water control Lane 11 to 13: Dellaportta. PCR product amplification size is 93 bp

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