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An Improved DNA Extraction Protocol from Four *in vitro* Banana Cultivars

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ABSTRACT

Molecular study of plants relies on high quality and yield of the DNA obtained. The leaf of banana contains high levels of polysaccharides, polyphenols and secondary metabolites. Hence, we developed an easy and efficient protocol for isolation of genomic DNA from leaves of four banana cultivars (Grand Naine, Poovan, Nendran and Red Banana). The method prevents solubilization of polysaccharides and polyphenols using high concentration of salt in combination with polyvinyl pyrrolidone and Cetyl trimethyl ammonium bromide. The purity of DNA was confirmed by 260/280 ratio from spectrophotometer readings and further by restriction digestion of the isolated DNA using *Hind III* and *EcoRI*. The yield of DNA ranged from 712-808 μg g^{-1} and the purity ratio was ranging from 1.7 to 1.8 showing minimal level of secondary metabolite contamination. Thus, this protocol proved amenable for PCR analysis and can be applicable to other plant related species also. Further more this protocol was suitable for randomly amplified polymorphic DNA analysis to study genetic variation in four banana cultivars.

Key words: Musa, polyphenols, polysaccharides, restriction digestion, polymerase chain reaction

INTRODUCTION

Banana belongs to the family Musa, which is considered as the country's prime fruit commodity. The FAO statistics denotes the world production of banana to be 70 million tones, of which India being the major producer (FAOSTAT, 2004). Banana industry is beset with low productivity due to various reasons like somaclonal variations and viral infections like Banana Bunchy Top Virus (BBTV), Banana Bract Mosaic Virus (BBrMV), Banana Streak Virus (BSV) and Cucumber Mosaic Virus. The cumulative damage caused by such viral infections could mount to be $4 billion by 2010, having a direct impact on the economical crisis (FAOSTAT, 2009). Hence, for successful implementation of crop improvement strategies, marker assisted selection, genotyping and genetic fidelity testing high quality genomic DNA is prerequisite. Polymerase chain reaction is a very powerful and useful technique used widely to study genetic make up of any organism. The PCR requires only minute quantity of DNA, but sensitive and accurate testing requires DNA in pure form free from polysaccharides.

DNA extraction from young leaves is mostly preferred compared to mature leaves as content of polysaccharides and polyphenols are less in young leaves (Zhang and Stewart, 2000). During

tissue homogenization, phenolics become oxidized and irreversibly bind to protein and nucleic acids (Loomis, 1974; Aljanabi *et al*., 1999) which become unsuitable for downstream work in molecular study (Qiang *et al*., 2004). Polysaccharides and polyphenols are particularly abundant in banana leaf samples and are not completely removed during classical extraction protocols; they remain as contaminants in the final DNA preparations. Polysaccharides make DNA viscous, glue like and non-amplifiable in the PCR reaction by inhibiting *Taq* enzyme activity and also interfere with accurate activity of restriction enzymes (Porebski *et al*., 1997).

Various protocols for DNA extraction have been successfully applied to many plant species (Doyle and Doyle, 1987; Ziegenhagen *et al*., 1993; Jayarama, 2009) which were further modified to provide DNA suitable for several kinds of analysis (Stewart and Laura, 1993; Ziegenhagen and Scholz, 1998). But these DNA extraction methods are generally expensive and time consuming. The need for a rapid and efficient method of DNA isolation for banana plant having high contents of polysaccharides is necessary when hundreds of samples need to be analyzed rapidly for virus indexing, variation studies and marker aid programs. Thus the protocol derived in this study is genus independent, efficient, inexpensive, simple and yields pure DNA amplifiable by PCR. The DNA which is isolated by the present protocol is suitable for further downstream applications.

MATERIALS AND METHODS

Plant material: Young leaves from *in vitro* micro propagated banana (Musa sp.) cultures (Red Banana, Nendran, Poovan and Grand Naine) were collected from Plant Tissue Culture Laboratory at Rise n'Shine Biotech Pvt. Ltd., Theur, Maharashtra, India. The leaves were brought to the laboratory in zip lock plastic bags packed in icebox which were immediately fixed in pre-chilled mortar and pestle using liquid nitrogen.

DNA extraction protocol: Three hundred microgram of fresh leaf tissues were ground to a fine powder using liquid nitrogen in prechilled mortar and pestle. Three milliliter of prewarmed extraction buffer (Tris-HCl-100 mM (pH 8), EDTA (Ethylenediaminetetraacetic acid)-20 mM (pH 8), 2% CTAB (Cetyl trimethylammonium bromide), 2% PVP (polyvinylpyrrolidone), 2% β-mercaptoethanol (v/v)) was added to the samples and it was ground once more in the buffer. All the samples were transferred to 1.5 mL microcentrifuge tubes, to which 10 μL Proteinase K was added. The tube was incubated at 37°C for 30 min and then at 65°C for another 30 min with frequent swirling. The samples were centrifuged at 8000 g for 10 min at RT and supernatant was transferred to fresh microcentrifuge tube. Equal volume of Chloroform and Isoamyl alcohol (24:1) were added and mixed by gentle inversion for 30-40 times. The samples were centrifuged at 8000 g for 10 min at RT and supernatant was transferred to fresh microcentrifuge tube. Two hundred microliter of 2 M NaCl containing 4% PEG 8000 (Hi media) solution was added. Incubate the samples for at least 15 min at 4°C to increase the recovery of DNA yield. To each tube add 2/3rd volume of isopropanol. The precipitated nucleic acid was collected and washed twice with wash solution (15 mM ammonium acetate in 75% of ethanol). The obtained nucleic acid pellet was air-dried until the ethanol was removed and dissolved in 100 μL of TE (10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8)) buffer with the addition of RNase (10 μL), incubated at 37°C for 30 min and stored at -20°C until use.

DNA analysis: The quality of extracted DNA was analyzed by means of agarose gel electrophoresis (0.8%), followed by ethidium bromide staining. The purity of the DNA was estimated

by spectrophotometer by calculating A260/280 and the yield was estimated by measuring absorbance at 260 nm. DNA purity was further confirmed by restriction digestion with two restriction enzymes, *EcoRI* and *Hind III* followed by gel electrophoresis. To check the suitability of extracted DNA for downstream analysis, RAPD analysis was done with OPC-02 (5'-GTGAGGCGTC-3') primer. Each PCR reaction mixture of 25 μL consisted of 100 ng genomic DNA, 2.5 μL of 10 X PCR reaction buffer, 2 μL of 25 mM $MgCl_2$, 0.5 μL of 2.5 mM dNTPs, 2 μL of 1 μM of primer (Bangalore Genei) and 1.5 U of Taq DNA polymerase (Invitrogen). The PCR amplification was performed in Eppendorf thermal cycler. The PCR reaction mixtures were heated at an initial step of 94°C for 4 min and then subjected to 40 cycles of following programme: 94°C for 1 min, 36°C for 1 min, 72°C for 2 min with a final extension temperature at 72°C for 10 min. The amplified DNA was electrophorosed on a 1.4% agarose gel containing 0.5 mg g^{-1} ethidium bromide and photographed in UV transilluminator. For comparison, the genomic DNA of same leaf sample was extracted using the existing CTAB protocol (Doyle and Doyle, 1987) and further DNA analysis was performed using same the conditions and reagents as described.

RESULTS AND DISCUSSION

DNA yield and purity: DNA extraction protocol using CTAB protocol (Doyle and Doyle, 1987) gave very low yield of DNA ranging from 341 to 440 μg g^{-1} (Table 1) across all four Banana cultivars (Red banana, Poovan, Nendran and Grand Naine). This yield was comparatively low to that of modified CTAB protocol where the DNA yield ranged from 712 to 808 μg g^{-1} (Table 2). The CTAB protocol revealed A260/280 ratio ranging from 1.5 to 1.6 (Table 1) when compared to modified CTAB protocol, where A260/280 ratio ranged from 1.7 to 1.8 (Table 2) indicating the absence of polyphenol contaminants. The purity of DNA was further confirmed by restriction digestion analysis using the enzymes *EcoRI* and *Hind III* (Fig. 1). In addition the smeared bands were observed on agarose gel which proved that the DNA extracted using CTAB protocol was contaminated with polysaccharides (Fig. 2) in comparison to modified CTAB protocol (Fig. 3).

PCR analysis: Further, DNA extracted using modified CTAB protocol maximally produced an average of 7 amplicons using OPC 02 (5'- GTGAGGCGTC -3') primer which were bright and reproducible (Fig. 4). This indicates that the DNA extracted by the modified CTAB protocol is

Table 1: DNA yield and purity, isolated from different banana cultivars by using CTAB protocol of Doyle and Doyle (1987)

Cultivar of banana	A_{230}	A_{260}	A_{280}	A_{260}/A_{230}	A_{260}/A_{280}	DNA concentration (μg g^{-1})
Nendran	0.0791	0.0690	0.0430	0.8723	1.6052	341.55
Poovan	0.0804	0.0830	0.0498	1.0323	1.6688	410.85
Red banana	0.1664	0.0900	0.0552	0.54086	1.6305	440.50
Grand naine	0.1073	0.0855	0.0546	0.7968	1.5656	423.23

Table 2: DNA yield and purity, isolated from different banana cultivars of by modified CTAB protocol

Cultivar of banana	A_{230}	A_{260}	A_{280}	A_{260}/A_{230}	A_{260}/A_{280}	DNA concentration (μg g^{-1})
Nendran	0.2057	0.1600	0.0871	0.7778	1.8361	792.00
Poovan	0.1554	0.1439	0.0816	0.9259	1.7630	712.31
Red banana	0.1195	0.1532	0.0827	1.2820	1.8534	758.34
Grand naine	0.1779	0.1633	0.0868	0.9179	1.8811	808.33

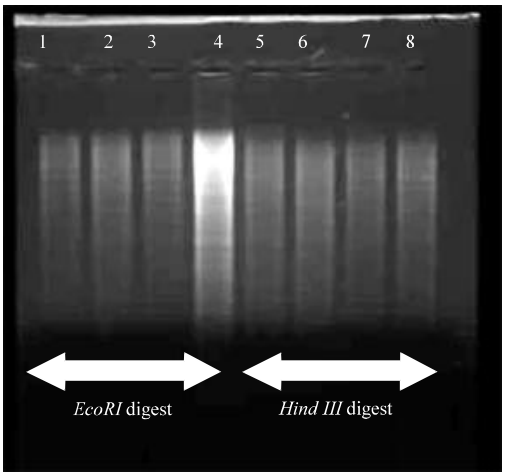

Fig. 1: Restriction enzyme digestion with *Eco RI* and *Hind III*. Lane 1-4: *EcoRI* digest of nendran, poovan, red banana and grand naine and Lane 5-8: *HindIII* digest of nendran, poovan, red banana and grand naine

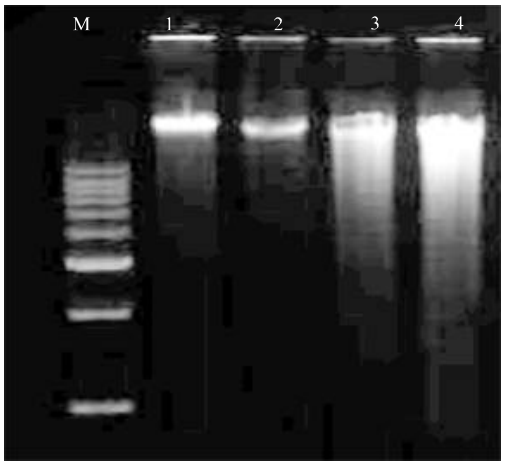

Fig. 2: DNA isolated by Doyle and Doyle (1987) protocol. Lane 1: Nendran; Lane 2: Poovan Lane 3: Red banana and Lane 4: Grand naine

suitable for PCR analysis whereas, DNA extracted using Doyle and Doyle protocol produced an average of 5.5 amplicons where the intensity of the band was very less and not reproducible (Fig. 5).

Compared with the CTAB method our modified CTAB DNA extraction protocol efficiently removed all polysaccharide contaminants. Published methods of DNA isolation from Doyle and Doyle (1987) proved unsuccessful and unreliable as the DNA obtained was with debris and highly viscous. This may be due to high levels of phenolics and polysaccharides in the samples (Sablok *et al*., 2009). In addition, the presence of polysaccharides in the DNA samples inhibits the activity of enzymes like polymerases, ligases and restriction endonucleases (Fang *et al*., 1992). Nucleic acids form a tight complex with the polysaccharides and are not available for reaction with above mentioned enzymes for further molecular study (Arun *et al*., 2002). The PVP forms complex

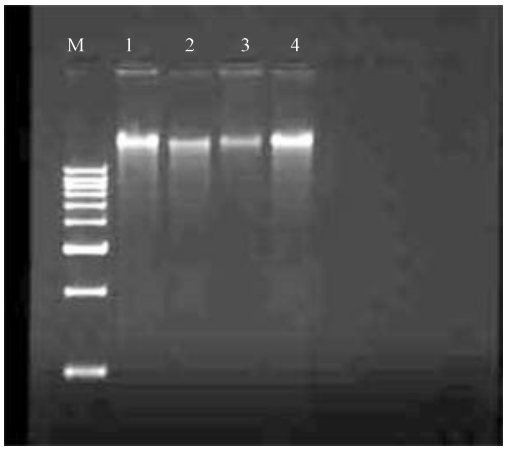

Fig. 3: DNA isolated by using modified CTAB protocol. Lane 1: Nendran; Lane 2: Poovan Lane 3: Red banana and Lane 4: Grand naine

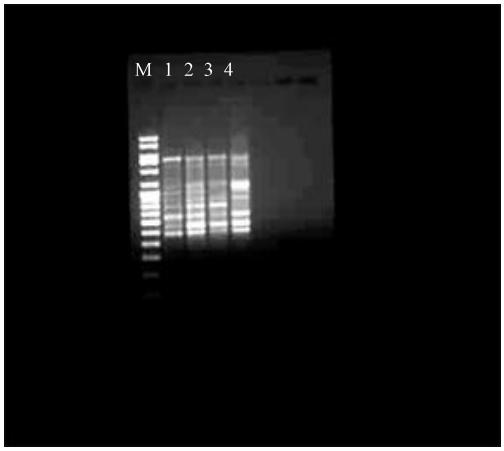

Fig. 4: RAPD assay conducted with the DNA isolated by means of modified CTAB protocol given in present study by primer OPC-02. Lanes 1- 4: Lane 1: Shows the amplification product belonging to Nendran, Lane 2: Poovan, Lane 3: Red banana and Lane 4: Grand naine

hydrogen binds with polyphenols and co precipitates with cell debris on lysis. When the extract is centrifuged in the presence of chloroform, the PVP complexes accumulate at the interface between the organic and the aqueous phases. The CTAB forms complexes with nucleic acids and proteins (Maliyakal, 1992). At high salt concentrations nucleic acids do not form complexes with CTAB and remain soluble in water, whereas complexes with proteins and polysaccharides are formed. These complexes are removed during subsequent chloroform extraction (Surzycki, 1999).

High salt concentration helps in the removal of polysaccharides (Aljanabi *et al*., 1999) which was evident with the results of the present study. Modified CTAB method (high alt ions and PVP) drastically decreased the yield of DNA (Narayanan *et al*., 2008) due to the lengthy and cumbersome and low temperature conditions steps involved for the removal of polysaccharides and polyphenols have lost much of DNA. This was in contradiction with the results of the present study where even though our modified CTAB method took 4 h for DNA isolation and the salt

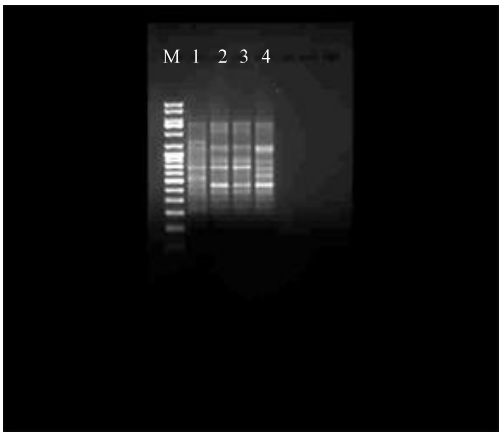

Fig. 5: RAPD assay conducted with the DNA isolated by means of Doyle and Doyle (1987) CTAB protocol by primer OPC-02. Lanes 1-4: Lane 1: The amplification product belonging to Nendran, Lane 2: Poovan, Lane 3: Red banana and Lane 4: Grand naine

concentration was high compared to CTAB method, the yield and purity of DNA was high. Lower number of amplicons in the extracted leaf sample using CTAB method suggests presence of contaminants like polysaccharides, polyphenols and RNA, which interfere with the activity of *Taq* polymerase (Scott and Playford, 1996). The time associated with DNA isolation and purification methods greatly influence the choice of the protocol, DNA yield and efficiency of PCR-RAPD amplifications (Ginwal and Mittal, 2010; Weishing *et al*., 1995). Quick DNA isolation protocols significantly influences the amplification pattern of RAPD markers due to high protein contamination (Narayanan *et al*., 2006). Our modified CTAB method took 4 h for extraction of high amounts of high quality genomic DNA compared to 3 h for CTAB protocol. Thus, the modified CTAB protocol inspite of its lengthy and cumbersome process yielded higher quality DNA suitable for molecular analysis and yield of the DNA.

The basic purpose of this study was mainly to identify and establish a very simple and easy DNA extraction protocol that can be applied to a larger scale DNA preparation also. The major highlight of this protocol were (1) High salt concentration was used to remove polysaccharides (2) PVP was used to remove phenolics compounds and proteinase K to remove proteins and (3) use of poly ethylene glycol to increase DNA recovery. This method can also be attempted to other crops where DNA extraction is difficult due to polyphenolic and polysaccharide contamination.

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