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AN EFFICIENT METHOD OF DNA EXTRACTION FROM *Theobroma cacao* AND *Cola nitida* LEAVES

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An efficient procedure for DNA extraction from recently matured green leaves of *Theobroma cacao* L. and *Cola nitida* is described. The procedure has been successfully used for extraction of good quality genomic DNA from cocoa accessions and the related genus, *Cola nitida*. It is a modified CTAB procedure, which includes use of high concentration of hexadecyltrimethyl ammonium bromide, (CTAB) (4%), and PVP in the extraction buffer to remove polysaccharides and polyphenols respectively. Addition of 5M NaCl during DNA precipitation with isopropanol, use of buffer in higher proportion in the extraction buffer, additional centrifugation steps during chloroform: isoamyl extractions, extended RNAse treatment, additional phenol: chloroform: isoamyl extractions and profuse additional washes of DNA pellet with ethanol to eliminate salt residues further helped in eliminating residual viscous substances and enhancing DNA recovery. The isolated DNA is of good quality and amenable to Polymerase Chain Reaction analysis.

Key words: CTAB, PCR, RAPD, DNA

Método eficaz para extração de DNA de folhas de *Theobroma cacao* e *Cola nitida*. É descrito um procedimento para extração de DNA de folhas de *Theobroma cacao* e *Cola nitida* em inicio de maturação. O processo tem sido usado com sucesso para extração de DNA genômico de boa qualidade de acessos de cacau e de cola. Trata-se do processo CTAB modificado, o qual inclui o uso de altas concentrações de bromohexadecil trimetil amônio (CTAB) a 4% e, PVP no tampão da extracão para remover polissacarídeos e polifenois, respectivamente. Adição de NaCl 5M com isopropanol durante a precipitação do DNA, uso de tampão em grande quantidade durante a extração do tampão, centrifugações adicionais durante a extração do clorofórmio: isoamil, extenção do tratamento com RNAse, adição de fenol: isoamil e lavados adicionais do "pelet" de DNA com etanol para eliminar os resíduos salinos, ao tempo que eliminam as substâncias viscosas residuais, facilitando a recuperação do DNA. O DNA isolado é de boa qualidade e apropriado para a análise de PCR.

Palavras-chave: cacaueiro, CTAB, PCR, RAPD, DNA

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Introduction

Cocoa (*Theobroma cacao* L.) is an important economical tropical beverage crop belonging to Sterculiaceae. DNA extraction from cocoa has been a difficult task. The leaves contain large quantities of polyphenols and polysaccharides (Figueira et al., 1992; Varadarajan and Prakash, 1991) and mucilaginous substances (Brooks and Guard, 1952), which turns difficult isolating good quality DNA. Initially, we tried extracting DNA from reported protocols such as Sagahai - Maroof et al., (1984), Dellaporta et al., (1983) and Doyle and Doyle (1990) using young and mature leaves. DNA isolated from the said protocols was highly viscous, brown and did not amplify by PCR (Table 1). We also tried DNA extraction from protocols reported for DNA extraction from cocoa. However, these protocols were expensive and tedious as they include isolation of crude nuclei (Perry et al., 1998) and sedimentation in Cesium chloride gradient centrifugation (Couch and Fritz, 1990, Figueira et al., 1992, Ronning and Schnell, 1995). Nuclear DNA extraction proposed by Crouzillat et al., (1996) and Tel-Azur et al., (1999) did yield good quality DNA, but the procedure is tedious, which involves several steps including filtration, use of separate lysis buffer and extraction buffer and Proteinase treatment. DNA extraction kits such as Qiagen Dneasy and Phytopure (Amersham) kits were quick and easy, but yielded less quantity of good quality DNA. Hence, our aim was to devise an alternate efficient protocol that could be easily carried out in moderately equipped laboratory without using sophisticated equipments. *Cola nitida*, the Cola nut is cultivated in tropical climates for edible nuts. Seeds contain caffeine and other chemicals, which have medicinal uses. There are no available reports of protocols for DNA extraction from Cola leaves.

Material and methods

Plant material

Fully grown, recently mature green leaves from field grown accessions were used in the experiment. Cocoa accessions and *Cola nitida* are maintained in the field gene bank at Central Plantation Crops Research Institute (CPCRI), Regional Station, Vittal, Karnataka, India. The germplasm maintained at CPCRI consists of introductions from Malaysia, Ghana, Nigeria and Kew gardens, UK. Four accessions of *Theobroma cacao* viz., I-14 (Malaysian accession), NC 25 and NC 27 (Nigerian accessions), SCA 12 and *Cola nitida* were used for standardizing the DNA extraction procedure. Leaves were kept in freezer (-20 °C) till use.

The protocol is adapted from that of Khanuja et al., (1999) with several modifications viz., use of buffer in higher proportion in the extraction buffer, additional centrifugation steps during chloroform-isoamyl extractions, extended RNase treatment, additional Phenol: chloroform extractions and profuse additional washes of DNA pellet with ethanol to eliminate salt residues.

Extraction buffer: 100 mM Tris HCl (pH 8.0); 25 mM EDTA; 1.4 M NaCl; 4% CTAB, 0.2% mercaptoethanol (v\v).

DNA Extraction Procedure

1. Wash freshly harvested leaves with cold water to remove debris and blot them dry.
2. Grind 1g leaf tissue (midrib removed) in liquid nitrogen and transfer to 50 mL polypropylene tubes.
3. Add 10mL extraction buffer to the ground leaf powder (1:10 tissue to buffer ratio).
4. Mix by inverting gently to slurry. Incubate at 60°C in a shaking water bath for 11/2 h.
5. Add 10 mL of Chloroform:Isoamyl alcohol (24: 1) and mix by inversion for about 15 min. Centrifuge at 13.400 xg for 10 min at 20°C.
6. Transfer the upper aqueous phase to an autoclaved tube. Repeat steps 5 and 6, 3-4 times till there in no white deposition at the interphase. Add 1.5 mL of 5 M NaCl and mix gently.
7. Add 0.6 volume of Isopropanol. Slow and careful mixing will result into floating fibrous DNA, which can be spooled and transferred into to 1.5mL micro centrifuge tube.
8. Centrifuge the DNA mass in microcentrifuge at high speed for 10 min at 20°C. Discard the supernatant
9. Wash the pellet thrice with 75% ethanol.
10. Air dries the pellet and dissolves in 100µ L TE buffer (10 mM Tris-Cl pH 8 and 1 mM EDTA).

Table 1. Comparative efficacy of CTAB protocols in genomic DNA extraction.

Protocols	DNA yield (µg/g) of fresh tissue	Amplification
Sagahai - Maroof et al., (1984)	18.66	No amplification
Doyle and Doyle (1990)	42	No amplification
Dellaporta et al., (1983)	63.43	No amplification
Porebski et al., (1997)	174	Amplified;inconsistent

DNA Enrichment / Purification

1. Add 6 µL of RNAase A (10 mg/mL) to 1 mL of DNA and incubate at 37 °C for one hour.

2. Extract with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by two extracts with Chloroform: Isoamyl alcohol (24:1). This step is repeated till there is no white deposition at the interphase.

3. Transfer the aqueous layer to a fresh 1.5 mL micro centrifuge tube and add 1/10 th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cøld ethanol. Spin in microcentrifuge at high speed for 10 min at 25°C.

4. Wash the pellet with 75% ethanol.

5. Dry the pellet and dissolve in 100µL of TE (10:1) buffer.

DNA Quantification

DNA concentration was measured by agarose gel electrophoresis (0.8%) by comparing the band intensity of an aliquot of the extracted DNA sample with known quantity of *Hind* III double digest. The purity of DNA was checked by recording the absorbance at A_{260} nm and A_{280} ratio in UV Spectrophotometer.

Polymerase Chain Reaction

PCR was carried out in 25 µL and amplification was performed in Eppendorf Mastercycler gradient Thermocycler. The PCR products were subjected to 1.4% agarose gel electrophoresis in 1x TAE buffer to separate fragments based on molecular weight. The amplified products were mixed with 2.5 µL loading buffer and loaded in each well of the gel. DNA standards 500bp (Bangalore Genei) were loaded on either end of the gel. Electrophoresis was carried out in TAE (40mM Tris-acetate, 1mM EDTA) buffer at 250 volts until the loading buffer reaches 1cm from the edge of the gel.

Results and Discussion

The method described here is efficient, which includes use of buffer in higher proportion, additional centrifugation steps during chloroform-isoamyl extractions, extended RNAse treatment for 1hr, additional Phenol: chloroform extractions and profuse additional washes of DNA pellet with ethanol to eliminate salt residues. The PVP freshly added at the time of grinding leaf tissue in liquid nitrogen effectively decreased polyphenolic content in the DNA extract. This protocol worked well with the related genus, *Cola nitida* leaves too. The recovered DNA from Cola leaves was amenable to PCR (Figure 1).

The average yield of unsheared high molecular weight DNA from this protocol was 18-40 mg/g of fresh weight of the fresh tissue. The molecular weight of genomic DNA was more than 20 Kb and the purity of DNA samples A_{260}/A_{280} ratio was more than 1.8 with minimum contamination by RNA. We attempted to optimize the stage of leaf tissue for DNA extraction, which is necessary to get the consistent PCR amplification. We used young, bronze colored, just mature light green and fully mature dark green leaves for the extraction. Very young bronze colored leaves produced highly viscous DNA containing polysaccharides. Such DNA could not be purified due to high viscosity. While mature green leaves yielded less amount of brown colored DNA, which is comparatively less viscous but contaminated with polyphenols. Due to contamination with polyphenols, the DNA isolates did not amplify by PCR. The polysaccharides and polyphenols form complex with DNA during extraction (Figueira et al., 1992) and DNA cannot be used for PCR and restriction analysis. Young, recent mature light green leaves were found to yield DNA with least contamination by polysaccharides and polyphenols.

Since cocoa is rich in polysaccharide content, tissue to extraction buffer ratio was increased to 1:10 proportion to dilute polysaccharide content and CTAB concentration in the extraction buffer was increased to 4% and this improved the quality of DNA. Earlier report on histochemical analysis indicates the ubiquitous presence of gum in lysigenous cavities in cocoa (Figueira et al., 1994).

Besides, we attempted to standardize the NaCl concentration in the extraction buffer to further eliminate polysaccharide content. The graded levels of NaCl viz., 1.0 M, 1.4 M, 2 M, 2.5 M, 3 M, 3.5 M and 4.0 M was used in the extraction buffer to test the optimum concentration. The quality of DNA recovered was good with 1.4 M and the DNA dissolved quickly in TE buffer. Whereas the DNA extracted using 1 M NaCl took long time to dissolve owing to impurities. Use of higher concentration of NaCl in the extraction buffer at 3.5 M and 4 M showed difficulty in the recovery of DNA. However addition of 5M NaCl along with isopropanol after chloroform; isoamyl alcohol extractions and just before DNA precipitation helped in the recovery of less viscous and clean DNA and the residual salt content associated with DNA pellet was removed by profuse ethanol washes (3-4 times). We also tried the efficacy of detergents using SDS and CTAB. CTAB was found effective in eliminating polysaccharides more effectively compared to SDS.

Amplification by PCR analysis: To evaluate the suitability of the isolated DNA to PCR and downstream applications, the DNA was amplified by PCR using random decamer primers (Figure 1 and 2). 30 ng DNA was sufficient for optimum amplification. Amplification was carried out in Eppendorf mastercycler gradient at 45°C annealing temperature.

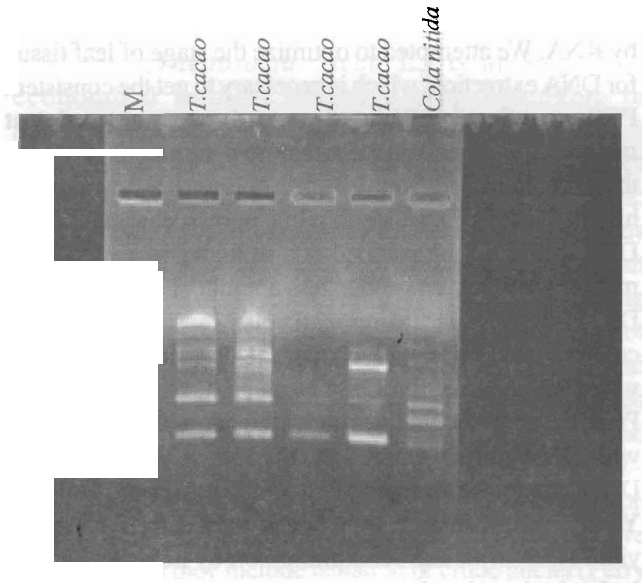

Figure1. PCR amplification profile of *Theobroma cacao* L. accessions and *Cola nitida* DNA using decamer primer, OPC 05. Amplification product was resolved on 1.4 % agarose stained with ethidium bromide. Lane 1-14, malaysian accession; Lane 2 - NC 27; Lane 3 - NC 25, Nigerian accession; Lane 4 - SCA 12; Lane- *Cola nitida*; M-Marker.

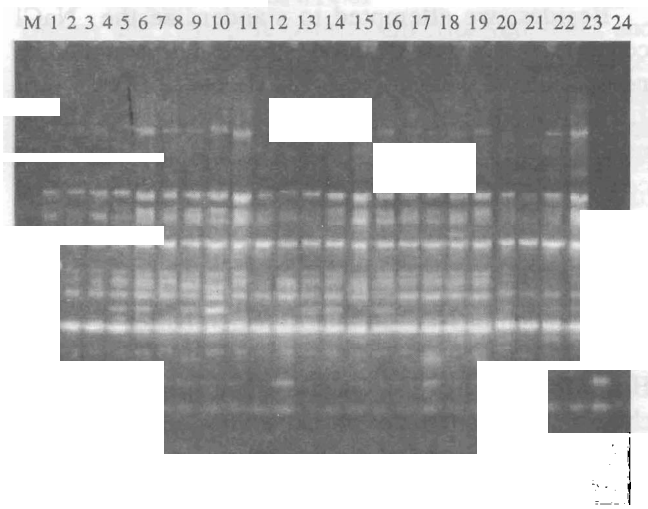

Figure 2. RAPD amplification of Nigerian cocoa accessions using OPC 18 primer. M- marker 100 bp. Lanes 1 to 24 represents DNA profiles of Nigerian accessions.

This protocol does not require proteinase treatment and ultra centrifugation using cesium chloride centrifugation. Good quality DNA in moderate quantities was isolated from this protocol, which is effective and can be successfully used for plants containing high resinous polysaccharides including related genus, *Cola nitida*.

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