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Rapid and efficient protocol for genomic DNA extraction from leaf tissues of coconut (*Cocos nucifera* L.)

Abstract

Good quality of the nucleic acid is the primary requisite for genomic research of crop plants. The presence of lipids, polysaccharides, polyphenols and protein molecules hinders downstream processes where genomic DNA has to be used as a template. Coconut leaf being highly fibrous and rich in all the secondary metabolites, isolation of good quality DNA remains a great challenge. Attempts to isolate the coconut DNA following the reported protocols are found not to yield DNA in the expected quality and quantity. A simple and fast approach for isolating the high-quality DNA from polysaccharides and polyphenolic-rich tissues of coconut is being detailed. As measured by its clear color, viscosity, and $A_{260/280}$ ratio, the isolated DNA was devoid of polysaccharides, polyphenols, RNA, and other significant impurities. In addition to the detailing of the modifications made in the CTAB method, this paper discusses the major step-by-step improvements among the widely-followed DNA isolation protocols.

Keywords: CTAB, molecular biology, molecular marker, nucleic acid isolation, palms, plant biotechnology

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Shri Hari Prasad, Lavale Shivaji Ajinath, Deepu Mathew
Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University, Thrissur – 680 656, India

Correspondence: Deepu Mathew, Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University, Thrissur – 680 656, India, Tel +919446478503, Email deepu.mathew@kau.in

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Introduction

Good quality macromolecules are absolute prerequisites in genomics and related researches. The presence of higher levels of contaminants such as polysaccharides, polyphenols, and proteins in the plant tissues makes the isolation of highly pure genomic DNA very difficult. This affects the downstream processes including restriction digestion, ligation, and thermal cycling,[1] necessitating the optimization of DNA isolation protocols for each species and even for each tissue.[2] Genomic DNA isolation is a tedious process in coconut since the leaves have higher phenolic and polysaccharide contents. Mechanical or physiological injuries to the tissues can trigger polyphenol release, leading to tissue browning.[3] The polyphenols undergo rapid oxidation and bind irreversibly to the DNA and proteins in the cell.[4] Brownish aggregates thus formed inhibit further enzymatic interactions and make the isolate unfit for further molecular analyses.

The isolation of coconut genomic DNA has been carried out mainly using CTAB[5] and Rogers and Bendich[6] protocols. Other methods initially developed for the plants such as French bean,[7-10] corn,[11,12] and date palm,[10,13] were also adopted in coconut, with varying levels of success. This paper details an efficient, cost-effective and rapid protocol to isolate good quality DNA with negligible polyphenol contamination.

Materials and methods

Optimization of the protocol

A thorough literature survey was made to understand each step in all the available DNA isolation protocols described in coconut and their advantages (Table 1). The most promising, reliable and preferred Cetyltrimethylammonium bromide (CTAB) method was considered for modifications to yield the highest amount of DNA.

Table 1 Comparison of different CTAB based DNA isolation methods

	Doyle and Doyle (1990)[5]	Križman et al. (2006)[14]	Ibrahim (2011)[15]	Murray and Thompson (1980)[7]	Saghai-Maroof et al. (1984)[8]	Porebski et al. (1997)[16]	Aljanabi et al. (1999)[17]	Angeles et al. (2005)[18]
Extraction buffer	CTAB (2%)	CTAB (2%)	CTAB (2%)	CTAB (1%)	CTAB (1%)	CTAB (2%)	CTAB (2%)	CTAB (2%)
	NaCl (1.4 M)	NaCl (2 M)	NaCl (1.4 M)	NaCl (0.7 M)	NaCl (0.7 M)	NaCl (1.4 M)	NaCl (2.2 M)	NaCl (2 M)
	β-mercaptoethanol (0.2%)	EDTA (20 mM)	β-mercaptoethanol (3%)	β-mercaptoethanol (1%)	β-mercaptoethanol (0.1%)	β-mercaptoethanol (0.3%)	β-mercaptoethanol (0.2%)	β-mercaptoethanol (0.2 M)
	EDTA (20 mM)	Tris-HCl (100 mM)	EDTA (20 mM)	EDTA (20 mM)	EDTA (10 mM)	EDTA (20 mM)	EDTA (50 mM)	EDTA (20 mM)
	Tris-HCl (100 mM)	pH 8.0	Tris-HCl (100 mM)	Tris-HCl (50 mM)	Tris-HCl (50 mM)	Tris-HCl (100 mM)	Tris-HCl (200 mM)	Tris-HCl (70 mM)
	pH 8.0	1 % PVP	pH 8.0	pH 8.0	pH 8.0	pH 8.0	pH 8.0	pH 8.0
		0.5 % activated charcoal before use	4 % PVP				Sodium sulfite (0.06%)	

Table Continued...

	Doyle and Doyle (1990)[5]	**Križman et al. (2006)[14]**	**Ibrahim (2011)[15]**	**Murray and Thompson (1980)[7]**	**Saghai-Maroof et al. (1984)[8]**	**Porebski et al. (1997)[16]**	**Aljanabi et al. (1999)[17]**	**Angeles et al. (2005)[18]**
Extraction	Preheat 5-7.5 mL of buffer in a 30 mL glass centrifuge tube to 60°C in a water bath. Powder 0.5-1.0 g fresh leaf tissue in liquid nitrogen in a chilled mortar and pestle.	Homogenize plant tissue in a mortar with 1.5 mL of extraction buffer. Transfer the mixture into a micro-centrifuge tube.	Extraction buffer was incubated in a water bath at 65 °C. Tissues ground to a fine powder under liquid nitrogen with the use of pre-chilled mortar and pestle.	Tissues ground using either mortar and pestle and glass beads or liquid nitrogen	Freeze-dried tissue was powdered with a mechanical mil, dispersed in 15 mL of extraction buffer.	Tissue ground using mortar and pestle with liquid nitrogen. Transfer frozen ground leaf tissue to centrifuge tubes. Add pre-heated extraction buffer and 50 mg PVP/0.5 g tissue.	Homogenize the fresh tissue with buffer (4 mL/g fresh tissue) using a homogenizer for a few seconds. Add 2 mL of 5% N-lauroyl-sarcosine, 2 mL of 10% PVP and 2 mL of 20% CTAB and mix by inversion.	Samples ground using mortar and pestle with liquid nitrogen and 500 mg of PVPP. Immediately transfer the ground samples to the tubes containing extraction buffer. Add 1 mL of 20% SDS and mix the contents.
Incubation	60°C for 30 min. with occasional gentle swirling.	Incubate the mixture at 55 °C for 30 min with frequent agitation.	15 µL of β-mercaptoethanol were added to the hot mix of CTAB extraction buffer and PVP, mixed well and was added to the frozen powder, mixed well and incubated at 65 °C for 30 min. with occasional mixing to avoid aggregation of homogenate.	Incubate at 50-60°C for 20-30 min, with occasional mixing.	Incubated at 60°C for 30-60 min with occasional mixing by gentle swirling.	Mix by inversion and incubate at 60°C (with shaking) for 25 to 60 minutes.	Incubate for 30-60 min at 65°C in a water bath with intermittent mixing.	Incubate the mixture at 65°C for 1 h.
Organic extraction	Single organic extraction. Extract once with chloroform: isoamyl alcohol (24: 1), mixing gently but thoroughly Centrifuge 6,000 xg for 10 min	Single organic extraction. Equal volume of chloroform: isoamyl alcohol to the supernatant and vortex thoroughly. Centrifuge at 16000 g for 10 min at room temperature. (repeat if cloudiness persist)	Repeated organic extraction. Equal volume of chloroform: iso-amyl alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min. The upper phase was carefully transferred to new sterile tube, one tenth volume of CTAB/NaCl(10% CTAB; 0.7 M NaCl) at 65 °C was added and mixed gently. One volume of chloroform : iso-amyle alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min.	Repeated organic extraction. Equal volume of chloroform: octanol (24:1) added, centrifuged at 13000 xg for 10 min. To the separated aqueous phase add one tenth volume of 10% CTAB, 0.7 M NaCl and chloroform/ octanol (24:1), centrifuged at 13000 xg for 10 min.	Single organic extraction. Chloroform: octanol (24:1) was added, and the solution was mixed by inversion to form an emulsion that was centrifuged at 5125 xg for 10 min at room temperature.	Repeated organic extraction. Six mL of chloroform: octanol (24:1) added and mix by inversion to form an emulsion. Centrifuge at 3000 rpm for 20 minutes at room temperature. Repeat chloroform-octanol extraction to remove cloudiness (PVP) in aqueous phase.	Single organic extraction. Equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol added mixed by inversion, centrifuged at 3000 g for 10 min at 4°C.	Not applicable
Precipitation	Remove aqueous phase to clean glass centrifuge tube, add 2/3 volumes cold isopropanol, and mix gently to precipitate nucleic acids After that, the sample was centrifuged at 10,000 rpm at 4°C for 10 min.	0.45 volume of isopropanol and mix by inversion. Incubate at 25°C for 1 hour. Centrifuged at 700 g for 10 min. at room temperature.	One volume of precipitation solution (1% CTAB; 50 mM Tris-HCl, 10 mM EDTA) was added, mixed gently and stored at -20 °C for 20 min. Centrifugation at 14,000 rpm for 5 min.	DNA precipitated using 2 mL CsCl gradients, mixed and centrifuged and visualized. Followed by removal of ethidium bromide and CsCl	Two-third volume of isopropanol was added to aqueous phase and mixed by two-to-four quick, gentle inversions.	Add half volume of 5 M NaC1 to the final aqueous solution recovered, mix well and add two volumes of cold (-20°C) ~95% ethanol. Mix by inversion. If required, place in -20°C for 10 min. (or 4 to 6 °C overnight). Centrifuge at 3,000 rpm for 6 min.	Add equal volume of isopropanol followed by 2 mL of 6 M NaCl. Incubate at -20 °C for at least 1 h.	Add 125 µL of 3 M sodium acetate and 500 µL of absolute isopropanol. Mix by inversion. Incubate at -80°C for 15 min, followed by centrifugation at 10,000 g at 4°C for 15 min.
Second precipitation	Not applicable	Not applicable	The pellet was carefully recovered and dissolved in 300 µL of high salt TE buffer. 200 µL of ice-cold Iso-propanol were added, mixed and followed by centrifugation at14,000 rpm for 10 min. The pellet was carefully recovered by decanting solution.	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable

Citation: Prasad SH, Ajinath LS, Mathew D. Rapid and efficient protocol for genomic DNA extraction from leaf tissues of coconut (*Cocos nucifera* L.). *Horticult Int J.* 2022;6(1):17–21. DOI: 10.15406/hij.2022.06.00236

Table Continued...

	Doyle and Doyle (1990)[5]	Križman et al. (2006)[14]	Ibrahim (2011)[15]	Murray and Thompson (1980)[7]	Saghai-Maroof et al. (1984)[8]	Porebski et al. (1997)[16]	Aljanabi et al. (1999)[17]	Angeles et al. (2005)[18]
Purification	If possible, spool out nucleic acids with a glass hook and transfer to 10-20 mL of wash buffer (76% EtOH, 10 mM ammonium acetate).	Wash the pellet by adding 1 mL of wash buffer (15 mM ammonium acetate in 75 % ethanol) and vortex. Centrifuge at 900 g for 10 min at room temperature.	The pellet was washed with 80% ice-cold ethanol, followed by 99.99% ice-cold ethanol.	Not applicable	DNA pellet purified using 76% ethanol/ 10mM ammonium acetate (NH_4OAc).	Wash pellet with 70% ethanol. Dry pellet in 37°C oven or vacuum until dry.	Wash the pellet using 70% ethanol, air dry	Pellet washed twice with 70% ethanol. Air-dry the pellet.
Resuspension	Resuspend nucleic acid pellet in 1 mL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).	Resuspend the pellet in 25 µL of TE buffer	Resuspend DNA pellets in 50 µL TE buffer.	Not applicable	Resuspend in 1.5mL of 10 mM NH_4OAc/ 0.25 mM EDTA	Resuspend in 300 µL TE (10 mM tris-HCl, 1 mM EDTA, pH 8.4) overnight	Resuspend pellet in 2-3 mL of TE (10 mM Tris- 1m MEDTA, pH 8.0).	Resuspend the pellet in 50 µL of sterile nanopure water

Chemicals

Extraction buffer (100 mL): 100 mM Tris-HCl, (pH 8.0), 20 mM EDTA (pH 8.0), 1.5 M NaCl, 2.0 % CTAB, 500 mg PVP, 1 mL β-mercaptoethanol.

Chloroform: isoamyl alcohol (24:1 ratio)

TE buffer (Tris 10 mM containing 1 mM EDTA)

RNase (10 mg/mL, place in a tube in a boiling water bath for 10 min., allow to cool on a bench and store at -20°C).

Improved protocol for DNA isolation

Leaf samples were collected from the emerging spear fronts of mature palms, brought to the laboratory in the icebox, frozen in liquid nitrogen, and stored at -20°C till further use. Following protocol was established to reduce the time required and for efficient use of chemicals in DNA isolation.

Frozen tissues were ground into fine powder in liquid nitrogen in an autoclaved pestle and transferred to 2.0 mL micro-centrifuge tube. The extraction buffer (1500 µL) was added to the microcentrifuge tube. The contents were homogenized and incubated for one hour at 65°C in a water bath, with occasional manual mixing by gentle swirling. After incubation, the contents were spun for 5 min. at 8000 rpm. About 750 µL of the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and the remaining cell debris were discarded. About 750 µL of chloroform: isoamyl alcohol (24:1) was added, mixed well and centrifuged for 10 min. at 13,000 rpm. This step was repeated twice by transferring the aqueous phase to a fresh 1.5 mL microcentrifuge tube, adding an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuging for 10 min. at 13,000 rpm. The aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube and an equal volume of isopropanol was added, mixed by gentle inversions and incubated at -20°C for one hour. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min. and the supernatant was gently decanted. DNA pellet was washed with 50 µL of 70 % ethanol by spinning at 8,000 rpm for 5 min., and tubes were kept inverted till the pellet got completely dried. Further, the pellet was dissolved in TE buffer (40-50 µL) and stored at -20°C.

Purification of extracted genomic DNA

DNA samples were treated with 2 µL RNase A solution (10 mg/mL) per 50 µL of TE and the tubes were incubated at 37°C in water bath for one hour. After the incubation, the temperature was increased to 65°C for 10-15 min. to denature the RNase A enzyme. An equal volume (~50 µL) of chloroform: isoamyl alcohol (24:1) was added, contents were mixed adequately, and centrifuged at 11,000 rpm for 5 min. The aqueous phase was transferred to a fresh 1.5 mL microcentrifuge tube. Equal volume of chloroform: isoamyl alcohol (24:1) added and the contents were centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to fresh 1.5 mL microcentrifuge tube, an equal volume of isopropanol was added, mixed by gentle inversions, and was incubated at -20°C for two hours. Tubes were centrifuged at 10,000 rpm for 10 min. and the supernatant was gently decanted. DNA pellet was washed with 50 µL of 70 % ethanol by spinning at 8,000 rpm for 5 min., followed by 100 % ethanol. It was dried and dissolved in TE buffer, and stored at -20°C.

Step-wise modifications in this protocol compared to the CTAB protocol, are presented in Table 2. In addition to the increased concentration of NaCl (1.5 M) and β-mercaptoethanol (1.0 %), PVP is also used in the new protocol. Incubation time for the ground samples is doubled and the organic extraction is repeated at least three times. For isopropanol precipitation, the new protocol uses incubation at -20°C for only one hour.

Table 2 Comparison of the modified CTAB method of DNA isolation and the Doyle and Doyle (1990) method

Parameter	Doyle and Doyle (1990)[5]	Modified in this work
Extraction buffer	CTAB (2.0 %)	CTAB (2.0 %)
	NaCl (1.4 M)	NaCl (1.5 M)
	β-mercaptoethanol (0.2%)	β-mercaptoethanol (1.0 %)
	EDTA (20 mM)	EDTA (20 mM)
	Tris-HCl (100 mM)	Tris-HCl (100 mM)
	pH 8.0	pH 8.0 500 mg PVP

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Table Continued...

Parameter	Doyle and Doyle (1990)[5]	Modified in this work
Extraction	Preheat 5-7.5 mL of extraction buffer in a 30 mL glass centrifuge tube, to 60°C in a water bath. Powder 0.5-1.0 g fresh leaf tissue in liquid nitrogen in a chilled mortar and pestle. Transfer the mixture into a micro-centrifuge tube add extraction buffer.	No change
Incubation	60°C for 30 min. with occasional mixing by gentle swirling.	60°C for 60 min with occasional mixing by gentle swirling
Organic extraction	Single organic extraction. Extract once with equal volume of chloroform: isoamyl alcohol (24: 1), mixing gently and centrifuge 6,000 xg for 10 min.	Repeated organic extraction. Extract with equal volume of chloroform: isoamyl alcohol (24: 1), mixing gently and centrifuge at 13000 rpm for 10 min.
Precipitation	Transfer aqueous phase to clean glass centrifuge tube, add 2/3 volumes cold isopropanol, and mix gently to precipitate nucleic acids. Centrifuged at 10,000 rpm at 4°C for 10 min.	Transfer aqueous phase to clean 2.5 mL micro-centrifuge tube, add equal volume of cold isopropanol, mix gently and incubated at -20°C for one hour. Centrifuge at 10,000 rpm at 4°C for 10 min.
Purification	If possible, spool out nucleic acids with a glass hook and transfer to 10-20 mL of wash buffer (76% EtOH, 10 mM ammonium acetate).	Pellet was washed with 50 µL of 70 per cent ethanol, and tubes were inverted till the pellet was air dried completely.
Resuspension	Resuspend nucleic acid pellet in 1 mL TE (10 mMTris containing 1 mM EDTA) and stored at -20°C	No change

Assessment of DNA quality

The quality and quantity of genomic DNA in each sample were determined using a NanoDrop spectrophotometer (ND-1000). Absorbance at 260 and 280 nm were recorded for each sample. The integrity of the DNA and presence of RNA or protein in the samples was assessed by electrophoresis in 0.8% agarose gel.

Result and discussion

To avoid the contamination by lipids, polyphenols and polysaccharides, the DNA was extracted from spear leaves. This tissue sample was preferred in earlier reports of DNA extraction.[12,19,20] Due to the presence of polyphenols, powdered leaf samples have instantaneously turned dark, after being frozen with liquid nitrogen. Also during the DNA extraction, a brown hue was seen in the heterogeneous isopropanol-DNA extraction buffer mixture.

Following the modified protocol, there was no evident discoloration of the pellets. This was due to increased concentration of β-mercaptoethanol. The use of a high concentration of β-mercaptoethanol is effective to remove the polyphenols.[21]

To combat phenolic compounds, a number of researchers have suggested using 2.0 % (w/v) low molecular weight (10,000 g/ Mol) PVP.[22,23] Low molecular weight PVP has a lower tendency to precipitate with nucleic acids than high molecular weight PVP, resulting in a sufficient amount of polyphenol-free DNA.[24] PVP has been used to isolate genomic DNA from other polyphenol-rich plants, including cotton,[23] sugarcane, lettuce, and strawberry,[17] grape, apple, pear, persimmon, and several conifers.[25]

The purity of genomic DNA measured as $A_{260/280}$ ratio has ranged from 1.78 to 1.84. $A_{260/230}$ ratio was <2 in all the samples, indicating that they are devoid of proteins and polyphenolic/ polysaccharide components.[8] The agarose gel profile was free of RNA and protein contamination and DNA bands were intact in all the samples (Figure 1).

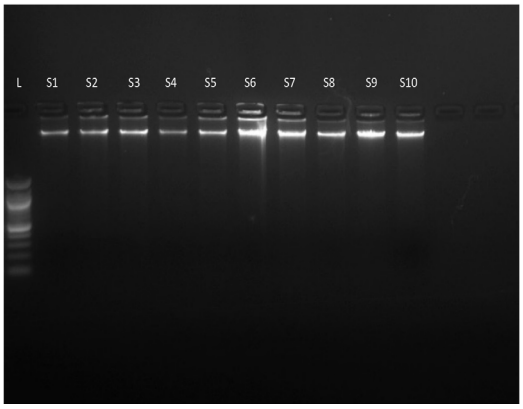

Figure 1 Genomic DNA isolated following the modified protocol. L: 100 bp ladder, S1-S10: DNA samples from different coconut accessions.

Conclusion

Through comparative analysis of the existing DNA extraction protocols, a modified CTAB method was suggested. Subsequently, this protocol was efficient to extract high-quality DNA from the polysaccharide and polyphenol-rich coconut leaves. Due to the simplicity and cost-effectiveness, this procedure is suggested for high-throughput sample preparations for genomic studies in coconut.

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Author contributions

SHP and DM has devised the new protocol, SHP and LSA performed the laboratory analyses, DM analysed the results, SHP and DM wrote and revised the manuscript.

Conflict of interest

Authors declare no conflict of interest.

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