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Isolation of genomic DNA from medicinal plants without liquid nitrogen

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Genomic DNA was extracted from eight medicinal plants using the present DNA extraction protocols (CTAB extraction method) with some modifications. Leaves were fixed in different fixing solutions containing absolute alcohol (99.99%), chloroform and EDTA, but without liquid nitrogen. DNA quality and quantity obtained were comparable to those isolated with liquid nitrogen, as the $\lambda_{260}/\lambda_{280}$ ratio with liquid nitrogen was in range 1.3-1.7 and with other fixing solutions it was 1.1-1.5. Absolute alcohol showed best results as fixing solution. Good quality of DNA was isolated without using liquid nitrogen from different medicinal plant species. DNA isolated by this method was suitable for various molecular biology applications.

Keywords: CTAB, DNA isolation, Liquid nitrogen, PCR, RAPD.

Search for an efficient means of extracting genomic DNA of high quality and yield has led to development of a variety of protocols. However, the fundamentals of DNA extraction remain the same. DNA must be purified from cellular material in a manner that prevents degradation. Biochemical composition in plant tissues of different species expected to vary considerably. Eight medicinally important plant species were selected for the present investigation, which are commonly found in semi-arid region of Rajasthan. These plants contain exceptionally high amounts of polysaccharides, polyphenols, and other secondary metabolites that have medicinal properties. Chemotypic heterogeneity among species may not allow optimal DNA yield with a single protocol. Thus, even closely related species may require different isolation protocols[1]. Various protocols for DNA extraction have successfully been applied to many plant species[2-5]. The major differences in these protocols mainly concern the ingredients and also *p*H of the extraction buffer. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA, while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB[6,7]. But liquid nitrogen can be difficult to procure in remote location. Thus, a method not requiring its use would be useful and economical for extraction of DNA. This can be done by fixing leaves in different solutions before CTAB DNA extraction, without using liquid nitrogen. DNA isolated by these methods is suitable for various molecular biology applications[8]. Genomic DNA was isolated from leaves of five species of stylo (*Stylosanthes scabra, S. seabrana , S. humilis* and *S. viscose*) using ethanol as leaf fixing solution instead of grinding in liquid nitrogen, which yielded high molecular weight DNA (30 kb). Isolated DNA was suitable for RAPD, sequence tagged site (STS) and restriction enzyme digestion[9]. Similarly DNA was isolated from leaves of ten plant species using three fixing solutions (alcohol; alcohol and chloroform; alcohol and EDTA)[8]. Isolated DNA was suitable for RAPD analysis, restriction digestion and cloning. Method was also developed for isolation of total cellular DNA from date palm (*Phoenix dactylifera L.*) that did not require liquid nitrogen. DNA was suitable for RFLP and PCR [10].

Materials and Methods

Plant material—Fresh leaves were collected from eight different medicinal plants selected from Tonk region (medicinal garden, Krishi Vigyan Kendra, Banasthali), *Azadirachta indica* (A. Juss.), *Ocimum sanctum* (Linn.), *Catharanthus roseus* (Linn.),

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Chlorophytum borivilianum (Sant. F), *Calotropis gigantean* (Linn.), *Syzygium cumini* (Linn.), *Zizyphus mauritiana* (Lam.), *Emblica officinalis* (Linn.).

DNA isolation—Fresh leaves of eight different plants were used in several treatment combinations to isolate total genomic DNA. Leaves were dipped in fixing solution for 30 min to denature enzymes at room temperature (RT) and at -80°C. The fixing solutions (5 ml/g tissue) were (A) absolute alcohol (99.99%), (B) alcohol-chloroform (70:30), and (C) alcohol–0.5 *M* EDTA (*p*H 8.0) (70:30). Treated tissue was removed from solution and homogenized with a mortar and pestle. Protocol was followed with slight modifications [different concentration of polyvinyl pyrrolidone (PVP; 1.5, 2.0 and 2.5%) and additional step with chloroform and iso-amyl alcohol (24:1)] in CTAB method[2] for efficient removal of polyphenol and polysaccharides (Table 1). DNA was diluted to 1000 times in Tris-EDTA buffer (*p*H 8.0) and quantified by taking the optical density (OD) at λ_{260} and λ_{280} with a spectrophotometer (Systronic UV-Vis 119). Took the readings at λ_{280} to obtain the ratio ($\lambda_{260}/\lambda_{280}$) as an indicator of DNA purity[11]. Purified DNA was observed on agarose gel (0.8%) after staining with ethidium bromide to ascertain its integrity.

RAPD analysis—RAPD analysis was done with six random primers using genomic DNA. After standardization, RAPD analysis was performed. PCR mixtures (25 µl) contained Taq buffer (2.5 µl), dNTPs (2.5 m*M*), primers (10 ng), *Taq* DNA polymerase (1U), and DNA (25 ng). The thermal cycler was operated — 1 cycle at 92°C for 1 min; 44 cycles at 92°C for 1 min, 48°C for 30 sec, and 72°C for 5 min; and a final amplification at 72°C for 2 min. Amplified fragments were separated on 1.8% agarose gel containing ethidium bromide.

Restriction analysis of DNA—DNA was restricted by *Eco*R I (Bangalore Genei) using 3 U/µg of DNA. The reaction mixture was incubated at 37°C overnight. Digested DNA was separated on agarose gel (0.8%), stained with ethidium bromide, and observed under UV light.

Table 1—Optimization of DNA extraction from *O. sanctum* using different isolation solutions

Isolation solution		$\lambda_{260}/\lambda_{280}$		DNA yield (µg/g tissue)	
		A	B	A	B
Without any modification in standard protocol		1.3	1.1	1060	1055
Different concentration of PVP	1.5%	1.4	1.2	120	1080
	2.0%	1.5	1.4	1430	1190
	2.5%	1.6	1.5	1550	1350
Repeated chloroform and iso-amyl alcohol extractions		1.6	1.5	1550	1350

A= DNA extraction with liquid nitrogen, B= DNA extraction without liquid nitrogen (absolute alcohol)

Results

High molecular weight genomic DNA resulted after fixing leaves in three solutions and isolating with CTAB method. For extracting purified DNA different concentrations of PVP were employed, but it yielded best at 2.5%. All the methods yielded good quality DNA (Fig. 1). In all the three fixing solutions, $\lambda_{260}/\lambda_{280}$ ratio was about 1.8 indicating the level of purity of DNA. DNA yield ranged from 1460-1820

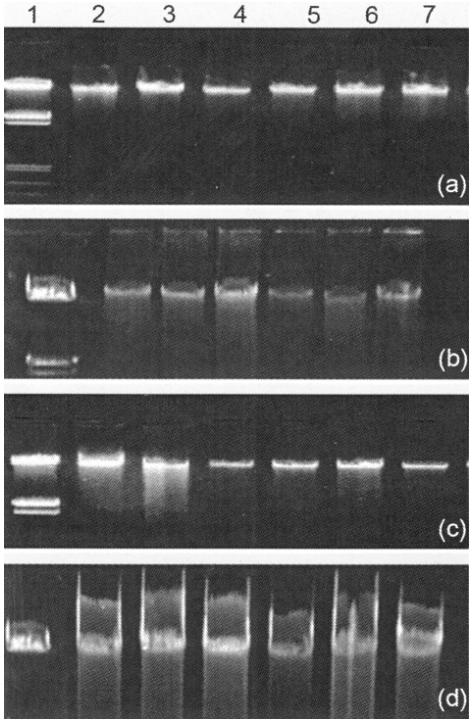

Fig. 1—Genomic DNA isolation from four different medicinal plants without using liquid nitrogen. (a) *Chlorophytum borivilianum*; (b) *Calotropis gigantean*; (c) *Azadirachta indica*; (d) *Catharanthus roseus*. [Lane 1 - λ DNA Eco R1/Hind III double digested; Lane 2 – After fixing leaves in alcohol at RT; Lane 3 – After fixing leaves in alcohol at -80°C; Lane 4 - After fixing leaves in alcohol and chloroform at RT; Lane 5 - After fixing leaves in alcohol and chloroform at -80°C; Lane 6 - After fixing leaves in alcohol and EDTA at RT and Lane 7 - After fixing leaves in alcohol and EDTA at -80°C]

µg/g of *Azadirachta* leaves and was comparable for the three fixing solutions (Fig. 2). Treatments applied to *Azadirachta* leaves at -80°C impaired DNA quality, as reflected by a $\lambda_{260}/\lambda_{280}$ ratio that was lesser at -80°C compared to room temperature (Table 2). Poor leaf DNA quality could be due to slow tissue death as a result of slow fixing solution penetration. In *Chlorophytum*, maximum amount of DNA (1066 µg/g) was obtained at room temperature when leaves were fixed in alcohol. Although good yield (883 µg/g) was also obtained in alcohol and chloroform treatment, it was lesser than yield obtained in alcohol:EDTA. In some plants alcohol and EDTA also gave good results (Table 3). Isolated DNA with different fixing solutions at different temperature was subjected to amplification with random primers. Amplified products were obtained in all cases. DNA isolated from *Azadirachta* with alcohol as fixing solution was amplified using 10 mer random primers (Fig. 3). A uniform pattern of bands was obtained by using different primers, which means that good quality DNA was obtained regardless of grinding method used. Genomic DNA was restricted by *Eco*R I (3U of enzyme/µg of DNA, kept overnight at 37°C). The restricted DNA produced smear on (0.8%) agarose gel, indicating complete digestion of DNA samples (Fig. 4).

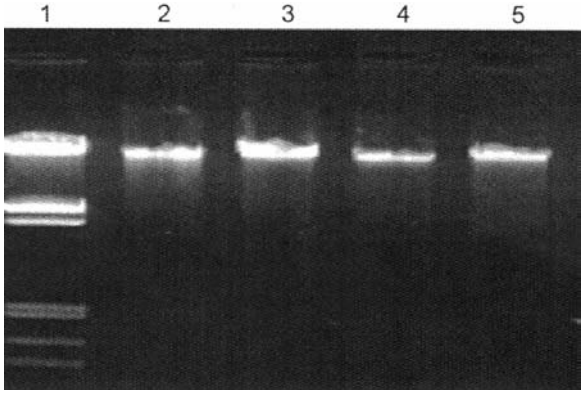

Fig. 2—Comparison of genomic DNA isolated without liq.N_2 and with liq.N_2 from two medicinal plants. [Lane 1- λ DNA *Eco R1/Hind* III double digested; Lane 2 - DNA isolated with liq. N_2 from *A. indica*; Lane 3- DNA isolated without liq.N_2 from *A. indica*; Lane 4- DNA isolated with liq. N_2 from *Emblica*; Lane 5- DNA isolated without liq.N_2 from *E. officinalis*] (Liq.–Liquid)

Discussion

Different fixing solutions used in the present study for isolating the genomic DNA from leaves of eight medicinal plant species showed difference in both quality and quantity of genomic DNA. In addition to the fixing of the leaves in alcohol, leaves were also ground in liquid nitrogen for comparison. The $\lambda_{260}/\lambda_{280}$ ratio with liquid nitrogen was in range 1.3-1.7 and with other fixing solutions it was 1.1-1.5. Thus, the results indicated not much difference in the quantity and quality of the genomic DNA isolated from the leaves either fixed in alcohol or ground in presence of liquid nitrogen. However, the comparable protocol as observed may lead in saving of liquid nitrogen when alcohol fixed tissues are used for DNA extraction, especially in those laboratories where availability of liquid nitrogen is a limiting factor. In turn, the procedure becomes economical too.

Three fixing treatments at room temperature (RT) for the rest of plants yielded comparable DNA quantities. All solutions were equally efficient in fixing leaves for DNA isolation. Addition of chloroform or EDTA to alcohol did not improve the fixation procedure, in fact they made the grinding of leaves difficult. So, alcohol treatment was, therefore, considered for further experiments and compared with the liquid nitrogen grinding method. The present procedure yielded high molecular weight DNA after grinding the leaves in liquid nitrogen as well as ethanol fixed leaves in CTAB. Thus, the results indicated not much difference in the quantity and quality of the genomic DNA isolated from the leaves either fixed in alcohol or ground in presence of liquid

Table 2—Genomic DNA yield obtained from *A. indica* leaves with three fixing solutions chilled at -80°C and room temperature (RT).

Treatments		OD_{260}	OD_{280}	$\lambda_{260}/\lambda_{280}$	Concentration (µg/µl)	DNA yield (µg/g tissue)
RT	A	0.182	0.146	1.25	9.10	1820
	B	0.166	0.146	1.14	8.30	1660
	C	0.162	0.142	1.14	8.15	1630
-80°C	A	0.164	0.151	1.09	8.20	1640
	B	0.147	0.132	1.11	7.35	1460
	C	0.150	0.119	1.26	7.50	1250

*DNA diluted a thousand times to measure OD
A = absolute alcohol, B = alcohol and chloroform, C = alcohol and EDTA

Table 3—DNA obtained from different plants by fixing leaves in different fixing solution at room temperature.

Plant species	Treatment	OD_{260}	OD_{280}	$\lambda_{260}/\lambda_{280}$	Conc. (µg/ µl)	DNA yield (µg/g tissue)
	A	0.163	0.112	1.46	8.10	1350
O. sanctum	B	0.224	0.151	1.48	11.20	1866
	C	0.313	0.202	1.55	15.65	2608
	A	0.203	0.136	1.49	10.15	1691
C. roseus	B	0.248	0.161	1.54	12.40	2066
	C	0.205	0.143	1.43	10.25	1708
	A	0.123	0.111	1.11	6.10	1066
C. borivilianum	B	0.105	0.093	1.13	5.2	883
	C	0.101	0.092	1.10	5.0	1666
	A	0.162	0.134	1.20	8.1	1350
C. gigantean	B	0.150	0.123	1.27	7.8	1300
	C	0.174	0.143	1.21	8.7	1450
	A	0.152	0.138	1.10	7.6	1266
S. cumini	B	0.151	0.137	1.10	7.5	1250
	C	0.119	0.106	1.12	5.9	983
	A	0.125	0.106	1.18	6.25	2083
Z. mauritiana	B	0.042	0.031	1.35	7.8	700
	C	0.131	0.114	1.15	6.5	1083

*DNA diluted a thousand times to measure OD
A = absolute alcohol, B = alcohol and chloroform, C = alcohol and EDTA

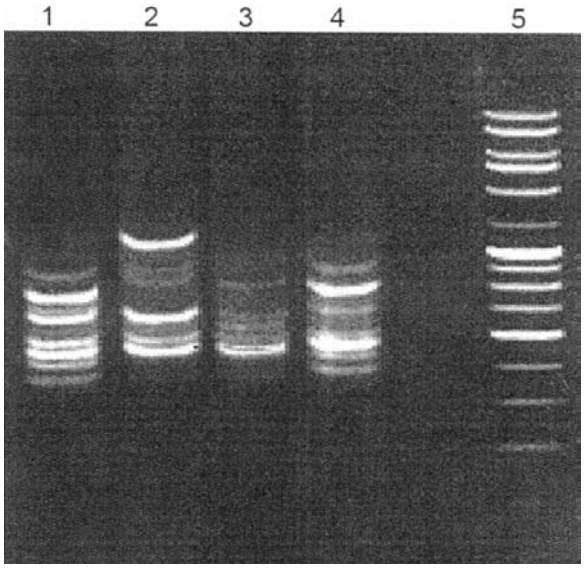

Fig. 3—DNA amplification pattern as observed for *A. indica* with different random primers (lane 1 and 4), 100 bp ladder (lane 5).

nitrogen. This genomic DNA obtained could be amplified using RAPD-PCR and good amplification was observed in case of all plants as shown in *Azadirachta* (Fig. 3). The results were in accordance to Sharma *et al.*[8], that good quality of DNA can be isolated without the use of liquid nitrogen from different plant species[8]. In some plant species $\lambda_{260}/\lambda_{280}$ ratio was less than 1.8 which indicated impaired DNA

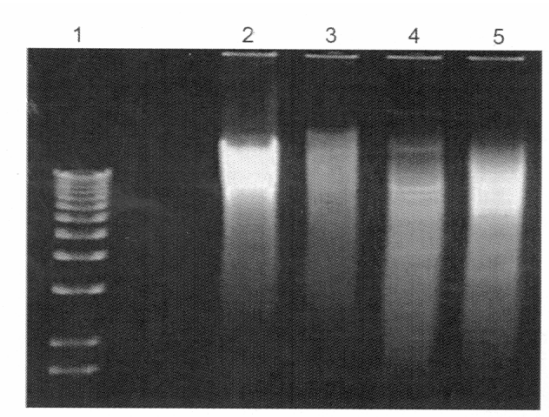

Fig. 4—*EcoR1* digested genomic DNA of *A. indica* and *E. officinalis* isolated with liquid nitrogen (lane 2 and 4), isolated by alcohol fixation (Lane 3 and 5), 100 bp ladder (lane 1)

quality. This may be due to the differences in levels of polysaccharides, fibers and associated anti-quality factors in these plant species. Advantage of the present protocol over existing methods is its simplicity, rapidity of isolation and reduction in the cost.

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