



Protocols

Isolating Plant Genomic DNA Without Liquid Nitrogen

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Abstract. DNA was isolated from leaves of 10 plant species (*Cuminum cyminum*, *Vigna aconitifolia*, *Pennisetum typhoides*, *Tecoma stans*, *Lycium barbarum*, *Anogeissus acuminata*, *Tecomella undulata*, *Zizyphus mauritiana*, *Phoenix dactylifera*, and *Eruca sativa*) and a fungus (*Fusarium oxysporum*) using the CTAB method. Three fixing solutions (alcohol, alcohol and chloroform, alcohol and EDTA) were used to produce high molecular weight DNA (>40 kb). DNA quality and quantity was comparable for the 3 fixing solutions and liquid nitrogen grinding. Adding chloroform or EDTA to fixing solutions offered no advantage over absolute alcohol. Isolated DNA was suitable for RAPD analysis, restriction digestion, and cloning. This method does not require liquid nitrogen for fixation, grinding, or storage at -80°C, making it advantageous over other common protocols.

Key words: cloning, CTAB, DNA isolation, genomic DNA, RAPD, restriction

Abbreviations: RAPD, random amplified polymorphic DNA; RT, room temperature.

Introduction

Intact high molecular weight plant nDNA is essential for molecular studies and genomic DNA library construction. Previously, tissues have been homogenized in buffers using a blender, mortar and pestle, or glass. These methods are limited because of degradation of DNA by DNases and other nucleases (Sharma and Sharma, 1980). The common procedure is to grind plant tissue in liquid nitrogen and transfer it to a preheated extraction buffer (Dellaporta et al., 1983; Saghai-Marouf et al., 1984; Mohapatra et al., 1992).

Liquid nitrogen can be difficult to procure in remote locations; thus, a method not requiring its use would be helpful. We have developed a protocol in which leaves are fixed in alcohol before CTAB DNA extraction, making liquid nitrogen unnecessary. DNA isolated by this method is suitable for various molecular biology applications.

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Materials and Methods

DNA isolation

The callus of cumin (*C. cyminum*) and young leaves of *C. cyminum*, *V. aconitifolia*, *P. typhoides*, *T. stans*, *L. barbarum*, *A. acuminata*, *T. undulata*, *Z. mauritiana*, *P. dactylifera*, *E. sativa*, and *F. oxysporum* were used in several treatment combinations to isolate total genomic DNA. The callus and leaves were dipped in fixing solution for 30 and 60 min to denature enzymes. The fixing solutions (5 mL/g tissue) were (A) absolute alcohol, (B) alcohol-chloroform (70:30), and (C) alcohol-0.5 M EDTA, [pH 8] (70:30). Treated tissue was removed from solution and homogenized with a mortar and pestle. Leaves were also homogenized in liquid nitrogen for comparison. Homogenized material was handled as follows (Mohapatra et al., 1992):

- Submerge 1 g of tissue in 5 mL of alcohol for 30 min.
- Allow alcohol to evaporate. Grind the tissue with a mortar and pestle.
- Transfer the homogenized tissue to prewarmed 2 X CTAB DNA extraction buffer (100 mM Tris [pH?], 20 mM EDTA [pH 8], 1.4 M NaCl, 2% CTAB, 2 μ L/mL β -mercaptoethanol).
- Incubate for 1 h in a 60°C water bath, occasionally mixing by gentle swirling.
- Remove from water bath. Add 0.6 vol of chloroform-isoamylalcohol (24:1). Mix by inversion for 15 min.
- Spin at 15,000 rpm [10,000 g] for 10 min.
- Transfer the aqueous phase to another tube.
- Add twice the volume of absolute alcohol or 0.6 vol of isopropanol to precipitate the DNA.
- Spool out or centrifuge briefly to pellet the DNA.
- Wash with 70% alcohol. Invert the tubes and drain on a paper towel for approximately 1 h. Dry overnight (cover with parafilm with tiny holes).
- Dissolve the dried DNA in T₁₀E₁ buffer (pH 8).
- Add 2.5 μ L of RNase to 0.5 mL of crude DNA (2.5 μ L of RNase = 25 μ g of RNase, thus treatment is at 50 μ g/mL of DNA preparation).
- Mix thoroughly but gently and incubate at 37°C for 1 h.
- Add 0.3-0.4 mL of chloroform-isoamylalcohol (24:1). Mix thoroughly for 15 min.
- Centrifuge for 15 min at 15,000 rpm [10,000 g].
- Remove the supernatant (avoid the whitish interface layer).
- Reprecipitate the DNA by using double the quantity of absolute alcohol.
- Remove the DNA with a pasture pipette or centrifuge the tube to pellet the DNA.
- Wash the pellet with 70% alcohol. Dry overnight.
- Redissolve the DNA in T₁₀E₁ buffer.
- Dilute the DNA 1000 times in T₁₀E₁ buffer and quantify by taking the optical density (OD) at λ_{260} with a spectrophotometer. Take readings at λ_{280} to obtain the $\lambda_{260}/\lambda_{280}$ ratio as an indicator of DNA purity (Sambrook et al., 1989).
- Observe purified DNA on 0.8% agarose gel after staining with ethidium bromide to ascertain its integrity.

RAPD analysis

RAPD analysis was done with 4 primers using 25, 37, and 50 ng of genomic DNA. After standardization, RAPD analysis was performed for the same one-plant samples subjected to different DNA isolation methods. The 25- μ L PCR mixture contained 2.5 μ L of buffer (10 X *Taq* DNA polymerase buffer containing 15 mM MgCl₂, Bangalore Genei, Bangalore, India); 2.5 μ M dNTPs (from 10-mM stock, Bangalore Genei); 15 ng primers (random decamer primer, Operon Technologies Inc. Alameda, California); 1 U *Taq* DNA polymerase (Bangalore Genei); and 25, 37, and 50 ng of DNA. The thermal cycler (Biometra) was operated as follows: 1 cycle at 92°C for 5 min, 37°C for 1 min, and 72°C for 2 min; 40 cycles at 92°C for 1 min, 37°C for 1 min, and 72°C for 2 min; and a final amplification at 72°C for 5 min. Amplified fragments were separated on 1.5% agarose gel containing ethidium bromide (0.5 μ g/mL), observed under UV light, and photographed using a Polaroid system.

Restriction analysis of DNA

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

Cloning DNA in *pBluescript SK2* \pm vector

Plant and vector DNA were digested with *Eco*R I. Generated fragments were reprecipitated after chloroform-isoamylalcohol (24:1) treatment. The vector and precipitated plant DNA fragments were mixed in a molar ratio of 1:8 for overnight ligation at 15°C using T4 DNA ligase enzyme (Bangalore Genei, Bangalore) in the buffer supplied by the company. Competent cells of *Escherichia coli* DH5 α were transformed with this ligated DNA sample. White colonies were selected on a media containing ampicillin, IPTG, and X-gal. Plasmid DNA was isolated following the standard alkaline lysis method (Birnboim and Dolly, 1979) and run on 0.8% agarose gel.

Results and Discussion

High molecular weight DNA (>40 kb, Figure 1) resulted after fixing leaves in 3 solutions—(A) alcohol, (B) alcohol and chloroform, (C) alcohol and EDTA—and isolating DNA with the CTAB method. The $\lambda_{260}/\lambda_{280}$ ratio was greater than 1.8 (ranging from 1.96-2.05), indicating DNA purity (Sambrook et al., 1989; Henry, 1997). DNA yield ranged from 272-360 μ g/g of cumint leaf and was comparable for the 3 fixing solutions (Table 1).

Tissue fixed at -80°C showed no difference in quantity or quality to DNA from tissue fixed at room temperature (RT). Fixing treatments were applied for different amounts of time. A treatment just sufficient to desiccate the leaves was appropriate and resulted in good quality DNA (data not shown). Treatments applied to cumint callus at -80°C impaired DNA quality, as reflected by a $\lambda_{260}/\lambda_{280}$ ratio less than 1.8. Poor callus DNA quality could be due to slow tissue death as a

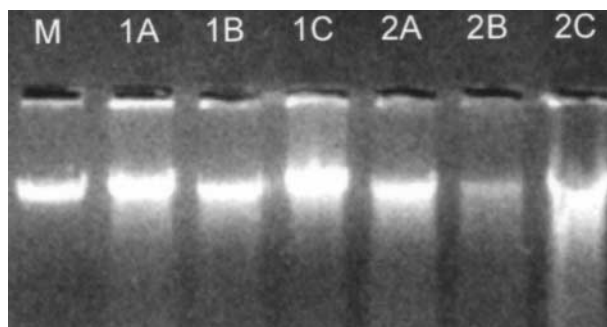


Figure 1. Cumin genomic DNA isolated by fixing leaves at (1) RT and (2) -80°C . M, uncut λ DNA (1 μg); A, alcohol; B, alcohol and chloroform; C, alcohol and EDTA.

Table 1. DNA yield obtained from cumin leaves with 3 fixing solutions chilled at -80°C and at RT.

Treatments		OD, λ_{260}	OD, λ_{280}	$\lambda_{260}/\lambda_{280}$	DNA* concentration, $\mu\text{g}/\mu\text{L}$	DNA yield, $\mu\text{g}/\text{g}$ tissue
RT	A	0.0660	0.0330	1.98	3.30	330
	B	0.0720	0.0350	2.05	3.60	360
	C	0.0588	0.0296	1.98	2.95	295
-80°C	A	0.0680	0.0336	2.02	3.40	340
	B	0.0695	0.0341	2.04	3.45	345
	C	0.0743	0.0379	1.96	3.72	372

*DNA diluted a thousand times to measure OD.

A = absolute alcohol, B = alcohol and chloroform, C = alcohol and EDTA.

result of slow fixing solution penetration. The 3 fixing treatments at RT yielded comparable DNA quantities in *Zizyphus* and *Tecoma* spp. (Table 2).

All solutions were equally efficient in fixing leaves for DNA isolation. Adding chloroform or EDTA did not improve the fixation procedure. Chloroform made the tissue slimy and slippery, making grinding difficult. EDTA delayed leaf desiccation. The alcohol treatment (A) was therefore considered for further experiments and comparison with the liquid nitrogen grinding method.

Alcohol-fixed leaves were ground and produced good quality DNA (255-520 $\mu\text{g}/\text{g}$) for different species of herbs and shrubs with the CTAB method (Table 3). Date palm leaves produced a poor DNA yield (48 $\mu\text{g}/\text{g}$) because their fibers made grinding difficult. Liquid nitrogen grinding was more successful for such fibrous materials. *Brassica* leaves yielded similar amounts of DNA when fixed in alcohol (376.75 $\mu\text{g}/\text{g}$) and ground in liquid nitrogen (370.5 $\mu\text{g}/\text{g}$) (Table 4). The $\lambda_{260}/\lambda_{280}$ ratio ranged from 1.83-2.00, indicating good quality DNA from both methods. We obtained DNA yield and quality similar to results reported with other protocols (Henry, 1997; Callahan and Mehta, 1991; Gawel and Jarret, 1991).

Table 2. DNA yield obtained from *Zizyphus* and *Tecoma* leaves fixed at RT.

Treatments		OD, λ_{260}	OD, λ_{280}	$\lambda_{260}/\lambda_{280}$	Concentration, $\mu\text{g}/\mu\text{L}$	DNA yield, $\mu\text{g}/\text{g}$ tissue
Zizyphus	A	0.0930	0.0500	1.860	4.65	465
	B	0.0875	0.0477	1.832	4.35	435
	C	0.0887	0.0506	1.752	4.43	443
Tecoma	A	0.0795	0.0396	2.006	3.97	397
	B	0.1079	0.0614	1.755	5.39	539
	C	0.0758	0.0404	1.875	3.80	380

*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 species by fixing leaves in absolute alcohol.

Species	OD, λ_{260}	OD, λ_{280}	$\lambda_{260}/\lambda_{280}$	Concentration, $\mu\text{g}/\mu\text{L}^*$	DNA yield, $\mu\text{g}/\text{g}$ tissue
<i>Anogeissus acuminata</i>	0.0514	0.0276	1.86	2.55	255
<i>Tecomella undulata</i>	0.1041	0.0539	1.93	5.20	520
<i>Phoenix dactylifera</i>	0.0096	0.0050	1.92	0.48	048
<i>Tecoma stans</i>	0.0795	0.0396	2.006	3.97	397
<i>Vigna aconitifolia</i>	0.0763	0.0385	1.98	3.81	381
<i>Lycium barbarum</i>	0.0632	0.0302	2.09	3.16	316
<i>Cuminum cyminum</i>	0.0880	0.0451	1.95	4.40	440
<i>Pennisetum typhoides</i>	0.0669	0.0332	2.01	3.34	334
<i>Brassica juncea</i>	0.0854	0.0441	1.93	4.27	427
<i>Eruca sativa</i>	0.0543	0.0278	1.95	2.71	271
<i>Fusarium oxysporum</i>	0.0684	0.0362	1.89	3.42	342

*DNA a diluted a thousand times to measure OD.

Table 4. DNA isolated from *B. juncea* varieties after fixing leaves in absolute alcohol (A) and grinding leaves in liquid nitrogen (B).

Varieties		OD, λ_{260}	OD, λ_{280}	$\lambda_{260}/\lambda_{280}$	Concentration, $\mu\text{g}/\mu\text{L}^*$	DNA yield, $\mu\text{g}/\text{g}$ tissue
BEC-114	A	0.0854	0.0448	1.90	4.27	427
	B	0.0784	0.0420	1.86	3.92	392
RN-505	A	0.0665	0.0332	1.88	3.32	332
	B	0.0803	0.0420	1.91	4.01	401
	B	0.0654	0.0354	1.84	3.27	327
Bio-902	A	0.0645	0.0352	1.83	3.22	322
	B	0.0725	0.0381	1.90	3.62	362
JM-1	A	0.0723	0.0366	1.97	3.61	361
	B	0.0682	0.0341	2.00	3.41	341

*DNA diluted a thousand times to measure OD.

A = alcohol, B = liquid nitrogen.

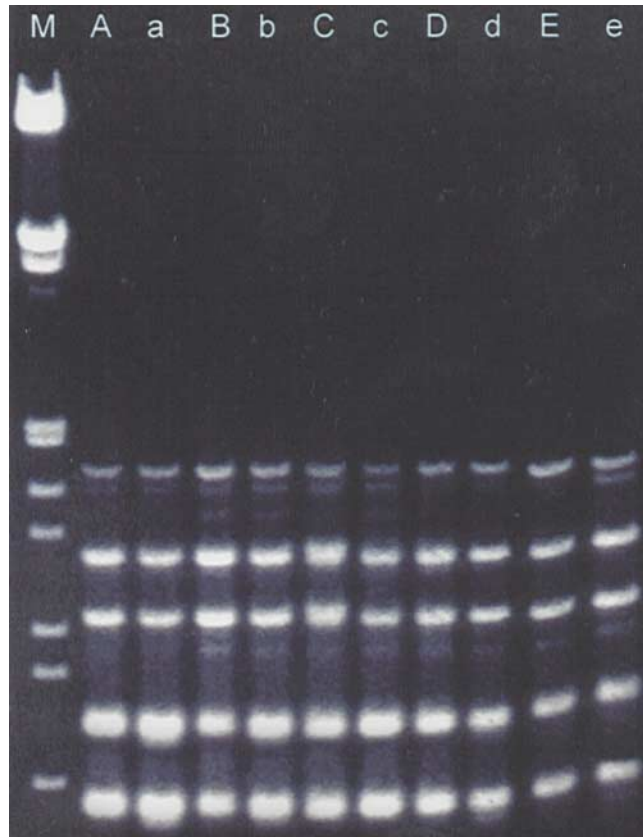


Figure 2. RAPD profiles generated for 5 *B. juncea* varieties (A-E) using primer OPC 9. M, λ DNA *Hind* III / *Eco*R I double digest; uppercase, DNA isolated by alcohol fixation; lowercase, DNA isolated by grinding in liquid nitrogen.

Random amplification of polymorphic DNA

DNA isolated from *C. cyminum* callus was amplified using 10mers, random primers (Operon Technologies Inc., USA). DNA isolated through 6 treatment combinations (A, B, and C solutions at RT and -80°C) was used for PCR amplification. DNA was diluted to $25\text{ ng}/\mu\text{L}$ in T_{10}E_1 buffer, and $1\ \mu\text{L}$, $1.5\ \mu\text{L}$, and $2\ \mu\text{L}$ of diluted DNA was used for amplification with 4 primers. When 25 ng of DNA was used, bands without upper DNA streaks were produced. A uniform banding pattern was expected because isolated DNA was from the same callus. Bands were uniform with all reactions, indicating good quality DNA. Genomic DNA isolated from 5 *B. juncea* varieties also produced uniform banding patterns, regardless of the grinding method used (Figure 2).

Restriction analysis of genomic DNA

Genomic DNA from 5 *Brassica* varieties was restricted by *Eco*R I ($3\ \text{U}$ of enzyme/ μg of DNA, kept overnight at 37°C). The restricted DNA produced a

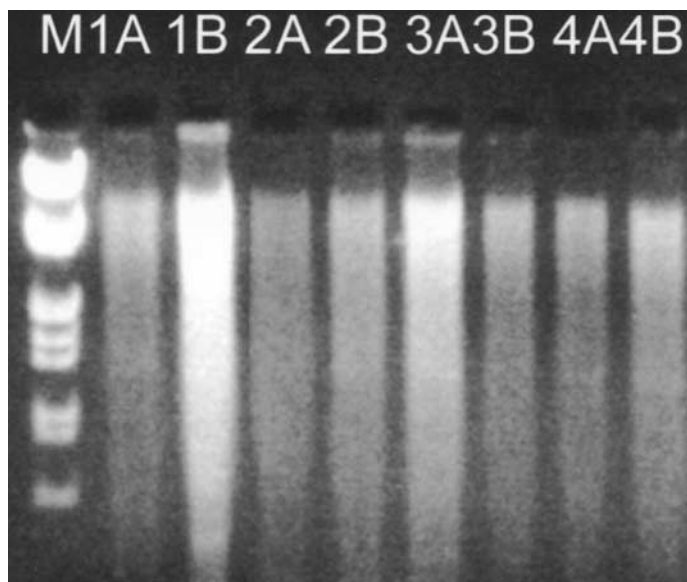


Figure 3. *EcoR* I cut genomic DNA of cumin (1-4) isolated by alcohol fixation (A) and by grinding leaves in liquid nitrogen (B). M, λ DNA *Hind* III / *EcoR* I double digest.

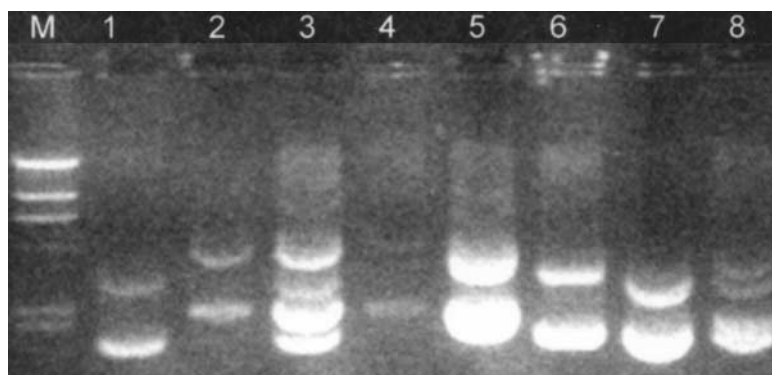


Figure 4. Recombinant clones carrying cumin genomic DNA fragments (lane 2-8). M, marker λ DNA *Hind* III digest; lane 1, original plasmid pBS.

good pattern on 0.8% agarose gel, indicating complete digestion of DNA samples from both methods (Figure 3).

Cloning of DNA isolated by alcohol fixation

Genomic DNA of *C. cyminum* and pBS (blue script Plasmid) restricted with *EcoR* I was purified using phenol-chloroform extraction for cloning. The vector and precipitated plant DNA fragments were mixed in a molar ratio of 1:8 for overnight ligation at 15°C using T4 DNA ligase enzyme. White colonies were selected from transformed *E. coli* DH5 α cultured in the presence of ampicillin, IPTG, and

X-gal. Selected colonies were cultured in Luria broth media overnight in the presence of ampicillin, and plasmid was isolated the following day. All colonies contained plasmids larger than pBS, confirming the presence of an insert (Figure 4). Thus, genomic DNA isolated by this procedure is suitable for cloning.

We conclude that genomic DNA can be easily extracted from plants by fixing leaves or other soft tissues in absolute alcohol without impairing its quality for routine molecular biological work. In our laboratory, we fix plant leaves in absolute alcohol and preserve them in paper bags for later DNA isolation. We do not depend on liquid nitrogen or a -80°C freezer to preserve live material for DNA isolation.

References

- Birnboim HC and Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA: a method for preparing plasmid DNA. *Nucl Acid Res* 7: 1513-23.
- Callahan FE and Mehta AM (1991) Alternative approach for consistent yields of total genomic DNA from cotton (*Gossypium hirsutum* L.). *Plant Mol Biol Rep* 9: 252-261.
- Dellaporta SL, Wood J, and Hicks JB (1983) A plant DNA miniprep: version II. *Plant Mol Biol Rep* 1: 19-21.
- Gawal NJ and Jarret RL (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. *Plant Mol Biol Rep* 9: 262-266.
- Henry RJ (1997) *Practical Applications of Plant Molecular Biology*, 1st ed, Chapman and Hall, London.
- Mohapatra T, Sharma RP, and Chopra VL (1992) Cloning and use of low copy sequence genomic DNA for RFLP analysis of somaclones in mustard (*Brassica juncea* [L] Czern and Coss). *Curr Sci* 62: 482-484.
- Saghai-Marouf MA, Soliman KM, Jorgensen RA, and Allard RW (1984) Ribosomal DNA spacer-length polymorphism in barley: mendelian inheritance, chromosomal location and population dynamics. *Proc Natl Acad USA* 81: 8014-18.
- Sambrook J, Fritsch EF, and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed, Nolan C (ed), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sharma AK and Sharma A (1980) *Chromosome Techniques: Theory and Practices*, 3rd ed, Butterworths Publishers, Fakenham, Norfolk.