ISOLATION OF GENOMIC DNA FROM FIVE SPECIES OF EUPHORBIACEAE WITHOUT LIQUID NITROGEN

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The isolation of intact, high-molecular mass genomic DNA is essential for many molecular biology applications including PCR, endonuclease digestion, southern blot analysis, and genomic library construction. Many protocols are available for the extraction of genomic DNA from plant tissue, and all the protocols have used expensive and hazardous chemical-liquid nitrogen for grinding plant material or DNA isolation kits for extraction. In the present study a modified CTAB (Cetyl trimethyl ammonium bromide) method has been used for isolation of genomic DNA from the leaves of five species of Euphorbiaceae viz., *Ricinus communis, Excoecaria robusta, Hevea brasiliensis*, *Jatropha multifida* and *Jatropha curcas.* Key step in the modified CTAB method is the use of alcohol for submerging leaf tissue instead of grinding it in liquid nitrogen. The protocol described (alcohol fixation) has yielded genomic DNA with excellent quality, which is comparable to those results of liquid nitrogen ground leaves. The genomic DNA isolated has an excellent absorbance (A_{260}/A_{280}) ratio observed between 1.80 and 2.00, indicating good genomic DNA without contaminants, highly suitable for PCR amplification.

Key words: CTAB; Euphorbiaceae; Genomic DNA; Latex-yielding plants; Without liquid nitrogen.

INTRODUCTION

DNA marker technology has been rapidly developing and many techniques that seemed unfeasible before are now routinely used. With the development of various molecular markers based on PCR technique such as RAPD, SSR, ISSR, STR, AFLP, and RFLP, molecular biology has greatly enhanced the speed and efficiency in crop improvement and breeding programmes, rDNA technology and construction of genomic DNA library. In general, it is difficult to extract and purify high-quality DNA from latex-yielding plants because of the presence of large quantities of secondary metabolites, polysaccharides, and proteins such as tannins,

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alkaloids and polyphenols. These compounds interfere by precipitating along with the DNA, thus degrading its quality and reducing the yield. Most extraction methods use expensive and hazardous chemicals for grinding plant tissue in liquid nitrogen to break down the cell wall of plants (Sharma *et al.* 2003) or freeze-drying (Michiels *et al.* 2003). Procurement and storage of liquid nitrogen may be difficult and expensive for many laboratories and transportation and handling of the same is also cumbersome. A good extraction procedure is considered to be one which results in DNA of reasonable purity without using the harmful chemical- liquid nitrogen. Thus, a method not requiring use of liquid nitrogen would be helpful to the researchers in remote areas. We have described here, an efficient protocol for isolating high-molecular PCR amplifiable DNA from leaves of five species of Euphorbiaceae viz., *Ricinus communis, Excoecaria robusta, Hevea brasiliensis*, *Jatropha multifida* and *Jatropha curcas.*

MATERIALS AND METHODS

The samples of young and tender leaves of five species of Euphorbiaceae viz., *R. communis, E. robusta, H. brasiliensis*, *J. multifida* and *J. curcas* were collected from the Botanical gardens of Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu. After washing the plant tissue with sterile water and subsequently with 70 per cent alcohol, 100mg of fresh leaf tissue of above species was taken and then it was chopped into fine pieces and subjected to genomic DNA isolation. Simultaneously leaves of same species were dried in an electric oven at 60° C for 24 hrs and 100mg of dry leaves were processed for extraction of genomic DNA. Genomic DNA was extracted from both fresh and dry leaves by adopting the CTAB method outlined by Doyle and Doyle (1990) without using liquid nitrogen. About 100mg of leaf tissue was submerged in absolute alcohol for an hour instead of grinding it in liquid nitrogen and put in a 2ml edppendorf tube. To grind the sample 1.5ml of CTAB extraction buffer (2% CTAB, 100mM Tris-HCl, 1.4M NaCl, 20mM EDTA di-sodium salt, 0.2M Mercaptoethanol 2% PVP pH 8.0) was added and incubated at 65° C for 60 min. Simultaneously liquid nitrogen ground leaves (fresh and dry) were also processed with CTAB buffer for comparison. The above mixtures were added with equal volume of chloroform: isoamylalcohol (24:1) and centrifuged at 5000rpm for 20min. After adding equal volume of cold isopropanol, the supernatant was stored at -20° C for an hour. Thereafter, the mixture was centrifuged at 3000rpm for 10min. The pellet was collected and treated with RNase (10mg/ml) for 30min. at 37° C. After an incubation period, 3M sodium acetate and absolute cold ethanol were added to the mixture and it was again incubated at –20° C for an hour and centrifuged at 3000rpm for 10min. After extracting the pellet with 70% ethanol twice it was air-dried and dissolved in 100µl of Milli Q water. The genomic DNA

isolated was quantified spectrometrically by measuring absorbance at 260nm and DNA was diluted to make a working solution for trial run of RAPD-PCR in thermal cycler.

RESULTS AND DISCUSSION

Genomic DNA isolated from fresh and dry leaves of five species of Euphorbiaceae using CTAB method with/without using liquid nitrogen are shown in Table 1. Genomic DNA yield from alcohol fixed fresh leaves ranged from 263 to 336 µg per gram fresh weight with A_{260}/A_{280} absorbance ratio between 1.80 and 2.00, indicating good genomic DNA free from polysaccharides, protein and secondary metabolites such as tannins, alkaloids, and polyphenols, whereas DNA isolated from dry leaves of alcohol fixed samples ranged from 187 to 256 µg per gram dry weight but A_{260}/A_{280} absorbance ratio was below 1.80, which ranged from 1.55 to 1.62, indicating high level of contamination with proteins, polysaccharides and polyphenols.

Table 1

Quality and quantity of genomic DNA isolated from fresh and dry leaves of *Ricinus communis, Excoecaria robusta*, *Hevea brasiliensis*, *Jatropha multifida* and *Jatropha curcas* using a modified CTAB method (without/with liquid nitrogen)

Species	Without liquid nitrogen (alcohol fixed leaves)				With liquid nitrogen			
	Fresh leaves		Dry leaves		Fresh leaves		Dry leaves	
	DNA yield (µg/g)	A_{260}/A_{280}	DNA yield (µg/g)	A_{260}/A_{280}	DNA yield (µg/g)	A_{260}/A_{280}	DNA yield (µg/g)	A_{260}/A_{280}
Ricinus communis	286±15.00^{ab}	1.90±0.10^{a}	187±17.00^{a}	1.58±0.04^{ab}	290±10.00^{b}	1.88±0.03^{ab}	126±11.00^{a}	1.62±0.10^{a}
Excoecaria robusta	286±25.00^{ab}	1.87±0.10^{a}	200±19.00^{a}	1.55±0.05^{a}	233±15.30^{a}	1.88±0.04^{ab}	196±15.00^{b}	1.66±0.08^{a}
Hevea brasiliensis	263±17.00^{a}	1.87±0.05^{a}	200±10.00^{a}	1.62±0.05^{ab}	233±20.00^{a}	1.83±0.02^{a}	193±17.00^{b}	1.63±0.10^{a}
Jatropha multifida	336±15.27^{c}	1.93±0.08^{a}	253±24.00^{a}	1.56±0.01^{a}	296±15.00^{b}	1.93±0.06^{a}	240±21.00^{b}	1.60±0.01^{a}
Jatropha curcas	320±20.00^{bc}	1.91±0.07^{a}	256±20.81^{a}	1.60±0.04^{ab}	283±20.81^{b}	1.88±0.04^{ab}	203±20.00^{b}	1.64±0.02^{a}

± = Standard Deviation (SD). Values followed by different letters (abc) are significantly different at α = 0.05% level according to Duncan's Multiple Range Test (DMRT) (n = 3 for all five species).

These compounds interfere by precipitating along with the DNA, thus degrading its quality and reducing the yield. In the present study, the purity of DNA isolated from alcohol fixed / liquid nitrogen ground dry leaves was however

questionable because of the presence of a high level of brown-coloured compounds which released upon cell lysis and irreversible adhered to DNA isolated, often reducing its quality and inhibiting PCR amplification.

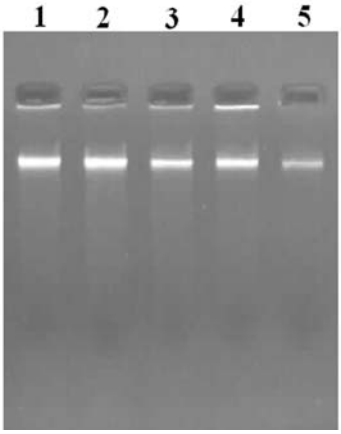

Fig. 1. Agarose gel Electrophoregram of DNA isolated from alcohol fixed fresh leaves of five species of Euphorbiaceae. (lane 1: *Ricinus communis*, lane 2: *Excoecaria robusta*, lane 3: *Hevea brasiliensis*, lane 4: *Jatropha multifida*, lane 5: *Jatropha curcas*).

The reason for this is that due to the cell death by drying in an electric oven, the dry leaves contain high levels of polysaccharides and polyphenolic compounds that are difficult to separate from DNA (Jobes *et al.* 1995, Ostrowska *et al.* 1998), but they are easily identified, because they make the DNA pellet sticky and gelatinous (polysaccharides) or often import a brown colour (polyphenols). In case of grinding the leaves in liquid nitrogen, the protocol described here yielded high molecular weight DNA which ranged from 233 to 296 µg per gram fresh weight with A_{260}/A_{280} absorbance ratio between 1.80 and 2.00. The highest yield of genomic DNA (336 µg) was recorded in the alcohol fixed fresh leaves of *J. multifida* which were comparable to those of genomic DNA isolated from liquid nitrogen ground leaves. Thus, the results obtained in the alcohol fixed and liquid nitrogen ground fresh leaves indicated not much difference in terms of quality and quantity of genomic DNA. Fixing the leaves in alcohol not only deactivates the enzymes, but also makes it more amenable to grind in the CTAB buffer. The alcohol fixed fresh leaves appear to be excellent for isolation of genomic DNA when compared to that of liquid nitrogen ground leaves.

This result is consistent with the results of Shahzadi *et al.* (2010), Chandra and Tewari (2007), Sharma *et al.* (2003), and Biswas and Biswas (2011). They observed high molecular weight DNA of large quantity and with good quality when a DNA extraction method using without liquid nitrogen was applied to fresh leaves of *Tagetes minuta*, *Stylo* sp., *Zizyphus mauritiana* and *Desmodium*

giganicum, respectively. Gel electrophoresis of the isolated genomic DNA further showed intact DNA bands without RNA and other contaminations (Fig. 1). The suitability of extracted DNA for downstream molecular processes was also verified by RAPD-PCR amplification. The genomic DNA of all five species studied was highly amplifiable by random primer (OPF-13) as indicated by the amplification products resolved on 1.5% agarose gel (Fig. 2). This further confirmed the purity of the DNA free from polysaccharide and polyphenol contamination which could otherwise inhibit the activity of Taq DNA polymerase in the PCR reactions.

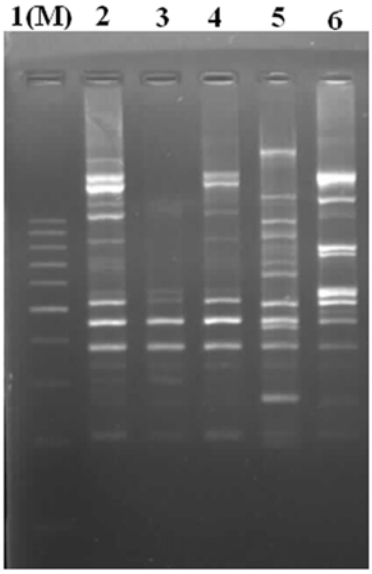

Fig. 2. RAPD profiles of DNA isolated from alcohol fixed fresh leaves of five species of Euphorbiaceae with primer OPF-13 (GGCTGCAGAA). (lane 1: 100 bp DNA ladder, lane 2: *Ricinus communis*, lane 3: *Excoecaria robusta*, lane 4: *Hevea brasiliensis*, lane 5: *Jatropha multifida*, lane 6: *Jatropha curcas*).

Although our experiments were carried out exclusively with five species of Euphorbiaceae, we believe that this modified CTAB method without using liquid nitrogen will be applicable to all types of tree species, though it is understood that some specimens or tree species will present their own specific isolation and purification challenges that might require modifications to this protocol. However, dipping the leaves in alcohol will be suitable for all tree species to extract DNA instead of grinding it in liquid nitrogen. In addition, this procedure is affordable and does not require sophisticated equipment making it a superior choice relative to expensive commercial kits for DNA isolation. Finally, we conclude that the PCR amplifiable high quality DNA can be easily extracted from fresh leaves of plant

species by fixing it in absolute alcohol without impairing its quality for routine molecular biological techniques.

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