EXTRACTION OF GENOMIC DNA FROM *JATROPHA sp.* USING MODIFIED CTAB METHOD

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Genomic DNA was isolated from leaves of three species of *Jatropha*, namely *J. glandulifera, J. gossypifolia* and *J. curcas.* The objective of our study was to use alcohol as a fixing solution, making liquid nitrogen unnecessary for isolation of genomic DNA from *Jatropha* species. The spectral quality of genomic DNA isolated using this method as measured by the A_{260}/A_{280} absorbance ratio ranged from 1.83 to 1.94 for all three species of *Jatropha.* DNA quality and quantity were comparable to those isolated with liquid nitrogen. The purity of the isolated DNA was further confirmed by PCR (RAPD) using 4 decamer primers. DNA samples prepared by this method were consistently amplifiable in the RAPD reaction and gave reproducible profiles. This method does not require for fixation or grinding in liquid nitrogen, making it advantageous over common protocol.

Key words: Biodiesel plant, Gel electrophoresis, Genomic DNA, *Jatropha curcas,* PCR, RAPD, simple DNA isolation method.

INTRODUCTION

The genus *Jatropha* that belongs to the Euphorbiaceae family is native of tropical America with more than 200 species that are widely distributed in tropics with a promise for use as an oil crop for biodiesel. With the application of molecular techniques in plant diversity conservation becoming increasingly popular, the isolation of impact, high-molecular mass genomic DNA becomes an important pre-requisite. However, species of *Jatropha* contain polysaccharides and polyphenols posing a major problem in the isolation of high quality DNA. Although several protocols are used for isolation of genomic DNA in *Jatropha* species by Ganesh Ram *et al.*, 2008; Ranade *et al.*, 2008; Pamidiamarri *et al.*, 2008 and Basha and Sujatha, 2007 and other species by Stein *et al.*, 2001; Dellaporta *et al.*, 1983; Sharp *et al.*, 1988; Murray *et al.*, 1980; Chunwongse *et al.*, 1993; McCarthy and Berger, 2002; Clarke *et al.*, 1989; Benito *et al.*, 1993; Krishna and Jawali, 1997; Hong Wang *et al.*, 1993; Lange *et al.*, (1998); Kamalay *et al.*, 1990; Francois Guidet, 1994 etc. all of them use expensive and toxic chemical liquid nitrogen. Moreover, no protocols used alcohol as leaf fixing solution instead of grinding in liquid nitrogen for isolation of DNA from *Jatropha* species. The presence of polyphenolics and polysaccharide content makes the isolation of high

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quality intact genomic DNA problematic. Although several successful DNA extraction protocols for plant species containing polyphenolics and polysaccharides compound have been developed, none of these are universally applicable to all plants (Varma *et al.*, 2007) and the published protocols are also limited because of degradation of DNA by DNases and other nucleases (Sharma and Sharma, 1980). Therefore, researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma *et al.*, 2007). A good isolation protocol should be simple, rapid and efficient, yielding appreciable levels of high quality DNA suitable for molecular analysis. The common procedure is to grind plant tissue in liquid nitrogen and transfer to a preheated extraction buffer (Dellaporta *et al.,* 1983; Mohapatra *et al.,* 1992). Liquid nitrogen can be difficult to procure in remote locations. Thus, a method not requiring use of liquid nitrogen would be helpful to researchers in remote area. In this paper we describe a DNA isolation method suited for isolation of genomic DNA in *Jatropha* leaves that can be stored for a longer duration, lasting for several PCR reactions. The method has used no expensive and toxic chemical. The aim to develop this method was to make this technique readily available in low-facility laboratories.

MATERIALS AND METHODS

Plant Material and DNA Extraction. Fresh leaves of *J. glandulifera*, *J. gossypifolia* and *J. curcas* were collected from our experimental field located at Annamalai University, Annamalainagar, Tamilnadu, India, and brought to the laboratory in polythene bags. The DNA was extracted from fresh leaves on the same day using modified CTAB method to obtain high quality intact DNA.

DNA Extraction Buffer and Chemicals. The CTAB extraction buffer used for the initial homogenization contained cetyl trimethyl ammonium bromide (CTAB) [2 % (w/v)], 100 mM Tris, pH 8, 1.4 M sodium chloride (NaCl), and 20 mM ethylene diamine tetra acetic acid (EDTA) di- sodium salt, pH 8.0. The extraction buffer was autoclaved and 2% polyvinylpyrrolidone (PVP), β-mercaptoethanol were added immediately before use. The chemicals and reagents used in the isolation of DNA were: chloroform: isoamyl alcohol (24:1, v/v); isopropanol and absolute alcohol. The washing solution contained 3 M Sodium acetate and 70% ethanol.

DNA Extraction Method. Fresh and young leaves were collected from *J. glandulifera, J. gossipyfolia* and *J. curcas*, to isolate genomic DNA. The fixing solution, namely absolute alcohol (Sharma *et al.,* 2003), was to isolate DNA without grinding the leaf samples in liquid nitrogen. Fresh leaves were submerged in absolute alcohol for 60 minutes to denature enzyme activity. For comparison, leaves were also homogenized in liquid nitrogen. 500 mg leaves of *Jatropha* species were submerged in 5 ml of absolute alcohol for 60 minutes. After removing

the leaves and alcohol allowed to evaporate, leaves were air dried to complete dry. The leaf tissues were ground in a preheated 2×CTAB extraction buffer. Liquid nitrogen ground samples were also processed with CTAB buffer. The samples were incubated for 60 minutes in 60° C water bath with occasional vigorous shaking. The samples were mixed gently after adding 500 µl of chloroform and isoamylalcohol (24:1) and placed on an orbit shaker for 20 minutes at room temperature. After centrifugation at 5 000 rpm, an equal volume of cold absolute isopropanol was added to the supernatant. The solution was mixed well and incubated for 60 minutes at 20° C. The sample was centrifuged for 5 minutes at 5 000 rpm to pellet the DNA followed by washing with 70 % alcohol and then allowed to drain dry. DNA was dissolved in 500 µl of double distilled water and 10 µl of RNase (10 mg / ml) and incubated at 37° C for 30 minutes. After incubation, 50 µl of 3 M sodium acetate and 1000 µl of absolute alcohol were added and incubated at –20° C for 60 minutes. The sample was centrifuged at 5 000 rpm for 10 minutes. The pellet was then washed with 70 % alcohol. After drying the pellet for 30 minutes at room temperature, DNA was resuspended in 50 µl of DNase free water.

Note: At this stage, the procedure may be stopped and the solution may be stored for several weeks at -20^{o} C.

DNA Quantification. After diluting the DNA two hundred times in DNase free water, it was quantified by taking the optical density (OD) at λ_{260} with a spectrophotometer. The purity of Genomic DNA was determined by the A_{260}/A_{280} absorbance ratio. The quality was also examined by running the extracted DNA samples on 0.8 % agarose gel stained with 10 mg/ml ethidium bromide in 1×TBE (Tris base, Boric acid, EDTA) buffer. The gel was visualized and photographed under UV light (Biorad).

PCR Amplification (RAPD). RAPD marker analysis for *J. glandulifera, J. gossypifolia* and *J. curcas* was performed in a final volume of 25 µl of reaction mixture containing approximately 50 ng of genomic DNA, 0.5U of Taq DNA polymerase (Sigma Aldrich Bangalore, India), 200 µm each of dATP, dCTP, dGTP and dTTP (Sigma Aldrich, Bangalore, India), 2.5 pmol. of primer (Sigma Aldrich), 2.5 mM of $MgCl_2$ and 10×PCR buffer. Amplification was carried out using an Eppendorf PCR thermal cylinder and programmed for 40 cycles of 94° C for 1 min, 35° C for 1 min, 72° C for 1 min, followed by a final extension step of 72 for 5 minutes. The amplification products were resolved on 2% agarose gel.

RESULTS AND DISCUSSION

Genomic DNA amplifications, southern blot analysis, AFLP, RFLP and DNA cloning necessitate the successful isolation of high quality DNA. To serve the purpose a DNA isolation protocol should ensure better quality and quantity at

low cost of production. Taking into consideration the limitation of liquid nitrogen in remote and less equipped laboratory, a method not requiring liquid nitrogen would be helpful and economical.

For the present method of genomic DNA isolation from the leaves of three *Jatropha* species, absolute alcohol was used for submerging leaf tissue instead of grinding in liquid nitrogen. Alcohol-fixed leaves ground in preheated 2×CTAB extraction buffer have yielded good quality of genomic DNA. In addition to the fixing of the leaves in alcohol, leaves were also ground in liquid nitrogen for comparison. Fixing of leaf tissue in alcohol not only deactivated the enzymes, but it has also made leaves more amenable to be ground in extraction buffer. Kumar *et al.* (2003) have reported that the presence of a high level of polysaccharides interferes with DNA isolation procedure and inhibits the activity of a wide range of DNA-modifying enzymes, such as restriction enzymes, polymerases and ligases. Chandra and Tewari (2007) concluded that the alcohol fixed leaves are useful for routine molecular biological work.

Genomic DNA isolated from the leaves ground in liquid nitrogen was observed comparable to those isolated with alcohol fixed leaves, thus avoiding the use of liquid nitrogen and in turn isolation of genomic DNA became economical.

The A_{260}/A_{280} absorbance ratio in alcohol fixed leaves (1.83 to 1.94) was more or less equal to nitrogen ground leaves (1.88–2.02) for *Jatropha* species indicating high purity of genomic DNA. DNA yield ranged from 2330–2710 µg/g when alcohol fixed leaves are used whereas 2260–2500 µg/g fresh weight when liquid nitrogen is used among three *Jatropha* species (Table 1). A ratio of absorbance (A_{260}/A_{280}) in the range 1.8–2.0 indicates a high level of purity (Pasakinskiene and Pasakinskiene, 1999).

Gel electrophoresis of the isolated DNA has further shown intact genomic DNA bands without RNA and other contaminations (Fig. 1). Kumar *et al.* (2003) and Richards *et al*. (1988) have reported that the presence of a high level of polysaccharides interferes with DNA isolation procedure and inhibits the activity of a wide range of DNA modifying enzymes such as restriction enzymes, polymerases and ligases. The polysaccharides were removed using an extraction buffer containing 1.4 M NaCl concentration. High ionic strength of CTAB forms complexes with protein and most of the acidic polysaccharides (Jones and Waker, 1963) where a high concentration of NaCl helps in the removal of polysaccharides (Aljanabi *et al.*, 1999). Fang *et al.* (1992) also observed that the addition of 1 M NaCl increases the solubility of polysaccharides in alcohol, effectively decreasing co-precipitation of the polysaccharides and DNA. CTAB binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction (Gawal and Jarret, 1991). This modified CTAB method appears to be excellent for the isolation of genomic DNA from *Jatropha* species. Phenolic content was removed using polyvinylpyrrolidone (PVP). PVP forms complex hydrogen bonds with latex lactones, lactucin and other phenolics and co-

precipitates with cell debris upon lysis. When the extract is centrifuged in the presence of chloroform, the PVP complexes accumulate at the interface between the organic and aqueous phases (Kim *et al.*, 1997; Maliyakal, 1992; Barnwell *et al.*, 1998; Michiels *et al.*, 2003). Dabo *et al.* (1993) concluded that photosynthetic active tissue contains phenolic compounds that oxidize during extraction and irreversibly interact with proteins and nucleic acids to form a gelatinous matrix. This matrix might inhibit proper extraction and amplification. We obtained DNA yield and quality similar to results reported with other protocols (Sharma *et al.*, 2003; Chandra and Tewari, 2007; Khan *et al.*, 2004).

Table 1
DNA yield obtained from leaves of *Jatropha* sps.

Soaked/ Ground in	Species	A_{280}	A_{260}/A_{280} ratio	DNA conc. (µg/µl)	DNA yield (µg/g tissue)
Alcohol	*J. glandulifera*	0.153 ±.012	1.836 ± .006	2.710 ± .085*	2710 ± 85
	J.gossipyfolia	0.127 ±.030	1.996 ± .045	2.333 ± .208*	2330 ± 208
	J. curcas	0.168 ±.055	1.943 ± .060	2.366 ± .152*	2360 ±52
Liquid nitrogen	*J. glandulifera*	0.133 ±.015	1.886 ± .065	2.433 ± .208*	2430 ± 201
	J. gossipyfolia	0.198 ±.131	2.026 ± .080	2.500 ± .100*	2500 ± 100
	J. curcas	0.114 ±.014	1.980 ± .020	2.266 ± .251*	2260 ± 251

DNA diluted two hundred times to measure.
* – indicates the plants are significantly different ($P < 0.05$)
± – Standard deviation

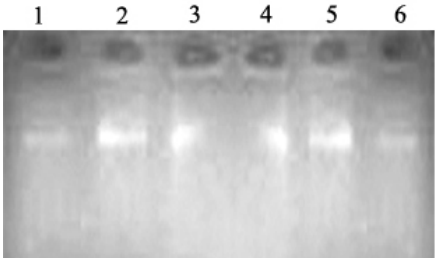

Fig. 1. Genomic DNA isolated by the modified CTAB method from fresh leaves *J. glandulifera* (Lane:1 & 4), *J. gossipyfolia* (lane 2 & 5), *J. curcas* (3 & 6), on 0.8% agarose gel. (Lane 1–3: Alcohol fixed leaves; Lane 4–6: Liquid nitrogen ground leaves).

DNA isolated from three species of *Jatropha* was amplified using four 10 mer- random primers (Table 2). DNA was diluted to 10 ng/µl in sterile water and used for amplification with four primers. With different random primers the total

number of bands amplified varied depending on the *Jatropha* species used for DNA isolation. A uniform DNA pattern of a species was expected when DNA was isolated either from ethanol or by using liquid nitrogen, because DNA was isolated from the same plants. Similarly uniform banding patterns were obtained when the experiment repeated indicating a good quality of isolated DNA. In general *J. gossipyfolia* produced a higher number of amplicons when campared to two other species, namely *J. glandulifera* and *J. curcas.* DNA isolated by this method from three species of *Jatropha* yielded clear banding patterns, as single band observed in *J. glandulifera* with primers OPM12 (Fig. 2, lane-4) and OPM13 (Fig. 3, lane-1) whereas three bands in other two species with primers OPM12 (Fig. 2, lane-5) and OPM13 (Fig. 3, lane-3) were produced. The maximum number of bands was observed in primers OPH18 and OPM14 for three species.

Table 2

List of primers used in RAPD analysis

S. No.	Primer Name	Sequence (5'-3'), length, method, annealing temp. °C
1.	OPH18	GAATCGGCCA, 10 mer (RAPD),35
2.	OPM12	GGGACGTTGG, 10 mer (RAPD),35
3.	OPM13	GGTGGTCAAG, 10 mer (RAPD),35
4.	OPM14	AGGGTCGTTC, 10 mer (RAPD),35

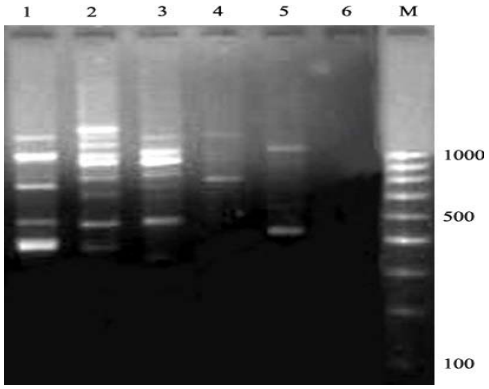

Fig. 2. Gel electrophoresis (2%) showing PCR profiles of amplified DNA from *J. glandulifera*, *J. gossipyfolia* and *J. curcas* (alcohol fixed leaves) using primers [OPM18 (lane 1– lane 3) and OPM12 (lane 4 – lane 6)], M: 100 bp Marker.

Basha and Sujatha, (2007) who isolated genomic DNA using standard CTAB method reported that RAPD marker showed a low level of molecular diversity among Indian accessions of *Jatropha* germplasm. Ganesh Ram *et al.*, (2008) extracted genomic DNA from leaves of *Jatropha* species by adopting the

procedure outlined by Dellaporta *et al.*, (1983) used to assess the genetic relationships between different *Jatropha* species using RAPD markers. RAPD analysis has been used for genetic diversity assessment and for identifying germplasm in a number of plant species. Ranade *et al.*, (2008) have also studied the diversity of *Jatropha curcas* using RAPD markers, who isolated genomic DNA using the DNeasy plant DNA extraction Kit (Qiagen, USA). The technical simplicity of the RAPD technique has facilitated its use in the analysis of the genetic relationship in several genera (Wilikie *et al.*, 1993; Nair *et al.*, 1999).

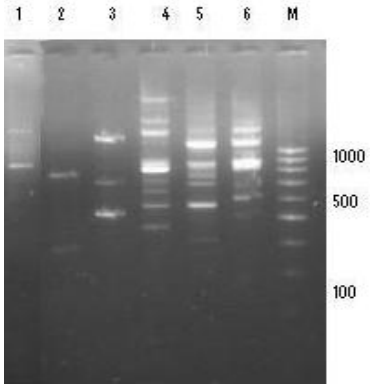

Fig. 3. Gel electrophoresis (2%) showing PCR profiles of amplified DNA from *J. glandulifera, J. gossipyfolia* and *J. curcas* (alcohol fixed leaves) using primers [OPM13 (lane 1 – lane 3) and OPM14 (lane 4 – lane 6)], M: 100 bp Marker.

CONCLUSION

No above protocol for DNA isolation from *Jatropha* has used the modified CTAB method and absolute alcohol as a fixing solution, making liquid nitrogen unnecessary. Taking into consideration the limitation of liquid nitrogen in remote area and less equipped laboratory, an effective procedure has therefore been developed for DNA extraction from *Jatropha*, which is a modification of the original CTAB method. The protocol described here is efficient, inexpensive, and yields clean genomic DNA, amplifiable by PCR, as indicated by the results of the RAPD technique. We do not depend on liquid nitrogen to grind leaf material for DNA isolation. This method is very simple and reliable for plant species like *Jatropha*.

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