RAPID DNA ISOLATION PROTOCOL FOR ANGIOSPERMIC PLANTS

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Summary. A simple, efficient, reliable and cost-effective method for isolation of total genomic DNA from both young and old leaves is described. The DNA obtained was free of any contaminating proteins, polysaccharides and coloured pigments. The isolated genomic DNA was found suitable for restriction digestion and PCR amplification, hence, it can be employed for preparation of Southern blots, AFLP and cloning. The protocol overcomes the need for liquid nitrogen, RNAase and phenol-chloroform treatment, usually employed for plant DNA isolation. In terms of quantity (up to 174 μg g^{-1} FW) and quality ($A_{260}/_{280}$ = 1.6 to 1.9) the present method has advantages over many other plant DNA isolation protocols. The protocol covers many and diverse angiospermic species belonging to different families within both dicotyledonous and monocotyledonous plants. However, the protocol could not be extended to gymnosperms.

***Key words*:** angiosperms, rapid DNA isolation.

***Abbreviations*:** CTAB – cetyltrimethyl ammonium bromide, EDTA – ethylenediamine tetraacetic acid, FW – fresh weight, SDS – sodium dodecyl sulphate, STE buffer – 400 mM sucrose, 2 mM Tris –HCl, pH 8.0, 20 mM EDTA, TE buffer – 10 mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0

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INTRODUCTION

A fast, simple, cost-effective and reliable method is a pre-requisite for any DNA extraction and subsequent downstream application. Many protocols have been used in plant DNA isolation, but because of the chemical heterogeneity of the species many of them could be applied to a limited number of species or even closely related species in some cases fail to respond to the same protocol (Weishing et al., 1995). Plants, especially medicinal plants contain an array of secondary metabolites. The compounds which make them interesting for molecular biology studies and hence, for DNA isolation, themselves interfere with the DNA isolation procedure. Another problem that could arise during plant DNA isolation is the necessity of liquid nitrogen for crushing the plant material as reported in most of the protocols (Ouenzar et al., 1998) and lengthy procedure involved. In many laboratories the availability of liquid nitrogen and RNAase is a limiting factor in DNA isolation. In the present study most of the concerns have been addressed. The protocol needs about an hour to prepare DNA for any molecular biology application. There is no need of liquid nitrogen during crushing the plant material. This method also bypasses RNAase and phenol-chloroform treatment. The present protocol can be employed for miniprep as well as maxiprep DNA isolations. The protocol was efficiently employed in 25 different plant species including both dicotyledonous and monocotyledonous plants including medicinally important species, such as *Withania somnifera* (Solanaceae*)*, *Tinospora cordifolia* (Menispermaceae), *Andrographis paniculat (*Acanthaceae)*, Pueraria tuberosa* (Paplionaceae), *Cymbopogon pendulus* (Poaceae), *Asparagus racemosus* (Liliaceae) and *Dioscorea composita (*Dioscoreaceae). However, we did not succeed to extend the protocol to gymnosperms. The method does not require expensive and hazardous reagents. It can be performed even in low technology laboratories. The quantity and the quality of the DNA extracted by this method is high enough to perform thousands of PCR- based reactions and can also be used in other DNA manipulation techniques, such as restriction digestion, AFLP, Southern blotting and cloning. The efficiency, speed and requirement of less expensive as well as non-hazardous chemicals make the present method an attractive alternative to the existing methods of genomic DNA isolations in plants. Using this protocol we were able to isolate DNA even from older leaves, which otherwise are recalcitrant to DNA isolation. The aim to develop this protocol was to make this technique readily available in low-facility laboratories and to minimize the duration of plant DNA isolation, as cumbersome procedures usually make the DNA prone to degradation.

MATERIALS AND METHODS

Plant material

To facilitate better homogenization leaf tissue was used for the experimental study. Keeping in view the criteria for the collection, fresh leaves were collected from the similar environmental conditions for DNA isolation studies. For comparing DNA concentrations in old and young leaves, the plant material was collected from the same plant. The material was sterilized with distilled autoclaved water and external moisture from the leaves was allowed to dry.

In the present study, young and old leaves were collected from four different plant species including three dicotyledonous plants (*Withania somnifera*, *Tinospora cordifolia*, *Pueraria tuberosa*) and one monocotyledonous plant (*Asparagus racemosus*).

Reagents and chemicals

The following chemicals and reagents were used: STE buffer (autoclaved) containing 0.4 M sucrose; 2mM Tris-HCl (pH8.0); 20 mM EDTA-Na_2; 20% SDS (autoclaved); 0.2% BSA; 0.2% β- mercapto ethanol; 8 M LiCl (autoclaved); chloroform: isoamyl alcohol (24:1, v/v); isopropanol and 70% ethanol.

DNA isolation protocol

500 mg of the leaf material was well homogenized in 10 ml of STE buffer containig 4% of SDS, 0.04% of BSA 0.2% β- mercapto-ethanol. SDS, BSA and β- mercapto-ethanol were added to the buffer immediately before use. The homogenization was done with sterilized mortar and pestle. LiCl (2 mM) was added to the homogenate before incubation at 65^0C for 30 min in a water bath. The incubated mixture was then left at room temperature for 10 min. Chloroform:isoamylalcohol (24:1, v/v) was added in an equal volume. The mixture was gently mixed and centrifuged at 15,000 rpm for 10 min at 4^0C. The upper phase was pipetted out in autoclaved centrifuge tube. The above step was repeated twice. Chilled isopropanol was added in a ratio of 2:3 to the separated phase. The suspended DNA threads were spooled out with the help of fine capillary. The DNA was then washed with 70% ethanol, dried in desiccator and finally resuspended in TE buffer (pH 8).

Estimation of DNA quality and quantity

Two DNA quantification methods were used. The first method was spectrophotometer measurement of UV absorption at 260 nm using Biophotometer (Eppendorf, Germany). The second method was the gel-method, which included ethidium bro-

mide fluorescence and lambda DNA as a standard, as described by Sambrook et al. (1989). The additional wavelength-related values of A_{230} A_{280} and A_{320} (recommended by Roger and Bendih,1985; Doyle and Doyle,1990; Wilkie**,** 1997) were also recorded.

PCR amplification

Polymerase chain reactions (PCRs) for amplification of DNA preparations were carried out in a 20 µl volume. The reaction tube contained 30 ng DNA, 1 unit of *Taq* DNA polymerase (Bio Basic, Canada), 2 mM of dNTP mix (Bio Basic Inc, Canada), 1.5 mM $MgCl_2$ (Bio Basic Inc, Canada) and 12 pmol of each decamer primer (Bio basic Inc., Canada). The amplifications were carried out using the Mastercycler gradient thermocycler (Eppendorf, Germany). The PCR programme consisted of 40 cycles, with an initial denaturation at 95^0C for 3 min. Each subsequent cycle comprised of 94^0C for 1min, 35^0C for 1min, 72 ^{0}C for 1min. A 10 min extension at 72^0C, followed the end of the programme cycle. The amplified assayed product was loaded in a 1.5% agarose gel containing 5 mg ml^{-1} ethidium bromide and subjected to electrophoresis at 100 volts. The gels were photographed by an Image Master VDS (Amersham Biopharmacia, USA). Primers were designed at RRL Jammu. They were designated as PL-136, PL-140, PL-151and PL-158, and had the following sequences:

PL-136: 5 $^/$-GGAGTACTGG-3 $^/$; PL-140: 5 $^/$-GGTCTAGAGG -3 $^/$,
PL-151: 5 $^/$-GAGTCTCAGG-3 $^/$; PL-158: 5 $^/$- GGACTGCAGA-3$^/$.

Restriction digestion

To determine the suitability of DNA for restriction digestion, 2.5 µg DNA was subjected to digestion with a number of enzymes viz., *Eco*RI, *Hind*III, *Not*I, *Alu*I and *Msp*I (Amersham Biopharmacia. USA). Incubation was performed overnight at 37^0C with 2 units of each enzyme. The digested DNA along with controls was subjected to electrophoresis in 0.8% agarose gel.

Results and discussion

The present protocol was applied to 25 species. Both young and older leaves responded to the protocol. However, the quantity was always less in the corresponding older leaves. The four plant species, which were taken for comparison of our results with those reported by Doyle and Doyle (1990) and Dellporta et al. (1983) showed higher DNA quantity in our protocol (Table 1). The A_{260}/ A_{280} values were in the range of 1.6-1.9. The DNA obtained was unshared, showing little or no RNA contamination (Fig.1). All tested samples showed amplification with given RAPD primers. The same was the case with the restriction digestion using the above-mentioned enzymes (Fig. 3, only restriction with *Hind* III is shown).

Table 1. Comparison of DNA quantity obtained following the present plant DNA isolation protocol with other routine methods.

S. №	Species	DNA (µg g^{-1} FW)					
		Present Method		Doyle and Doyle (1990)		Dellaporta *et al.*, (1983)	
	Type of the tissue	Young leaves	Old leaves	Young leaves	Old leaves	Young leaves	Old leaves
1.	*Withania somnifera*	106-143	47-58	37-45	6-13	27-35	4-13
2.	*Tinospora cordifolia*	135-174	45-69	42-63	17-23	23-44	9-21
3.	*Pueraria tuberosa*	109-147	65-96	29-48	25-36	12-28	5-17
4.	*Cymbopogon pendulus*	67-105	33-58	41-58	11-24	12-19	3-7

Genomic DNA amplifications, Southern blot analysis, AFLP 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 in a shorter period of time. Secondly, a DNA isolation protocol should be extendable to a wide diversity of plant species and should also be applicable for high throughput for routine DNA studies in low facility laboratories. In the present study, a number of principles were applied to simplify the DNA extraction procedure that did not adversely affect the DNA quality and quantity. The present protocol does not include the need for liquid nitrogen for crushing the plant material. A similar procedure for isolation of DNA from date palms has been followed by Ouenzar et al. (1998). However, instead of mannitol and polyethylene glycol (PEG 6000), which did not give consistent results, we used sucrose in the extraction buffer. Sucrose has been used in the extraction buffer by Kaufman et al. (1999), Buldewo and Jaufeerally-Fakim (2002) for total DNA isolation from plant cells, Willmitzer and Wagner (1981) for isolation of nuclei from plants and Giordano et al. (1999) for the extraction of DNA from a fungus. Sucrose has nevertheless been found to stabilize proteins in *in vivo* systems (Lee and Timasheff, 1981), but the effect may not be extrapolated to *in vitro* conditions keeping in view the role of other chemicals like EDTA, b- mercapto-ethanol and SDS. Sucrose has also been found to enhance thermostability of BSA (Baier et al., 2001). Although the role of sucrose in the DNA isolation is not conclusive, we found that its omission in the lysis buffer adversely affected DNA yield in 50% of the cases. Addition of b-mercapto-ethanol inhibited polyphenol oxidation and thus subsequent browning of the DNA samples. Besides breaking the cell wall and the nuclear membrane, SDS inhibits nucleases and helps separate the proteins from the nucleic acids (esp. in ribosomes). In the present method 30 min incubation at 65ºC was found necessary to obtain optimum results.

The advantages of our method are evident when comparing our method with the routine CTAB and SDS methods (Table1). Far high quality of DNA (>150 mg g^{-1}

FW) was obtained. In the usual CTAB protocol and the protocol described by Kaufman et al. (1999) and Buldewo and Jaufeerally-Fakim (2002), it takes considerable time and effort to obtain the equivalent quantity of DNA.

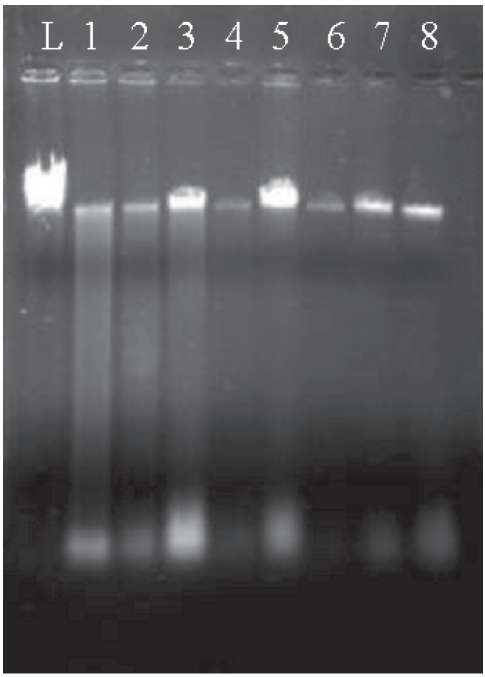

Fig 1. DNA isolated from leaves of four plant species (both young and old leaves, respectively) resolved on 0.8% agarose gel. 'L' represents uncut Lambda phage DNA. Lane 1 and 2: *Withania somnifera*, Lane 3 and 4: *Tinospora cordifolia*, Lane 5 and 6: *Pueraria tuberosa*, Lane 7 and 8: *Cymbopogon pendulus*

The A_{260}/A_{280} values were found within the acceptable range of 1.6 -1.9 (data not shown). The A_{260}/A_{280} and A_{260}/A_{230} ratios indicate the protein, polyphenol and carbohydrate contaminations, respectively (Manning 1991). The absorbances at 230, 260 and 280 nm were within the recommended ranges (Roger and Bendih 1985; Doyle and Doyle 1990). LiCl was used as described by Pirttila et al. (2001) and as it is known it binds more specifically to RNA. The Li-RNA complex is insoluble and thus gets efficiently precipitated, except for molecules smaller than 200 bases. In our study a little amount of RNA (appr. 200 bases) was usually obtained (Fig.1), but it did not show any effect on molecular biology applications. In addition, LiCl precipitates also selectively shared DNA, residual proteins and neutral polysaccharides. The removal of RNA by LiCl was better facilitated by keeping the mixture in cold for some time, even though several workers have indicated that there is no effect of cold treatment on RNA precipitation with LiCl. However, it was found advantageous that while using SDS along with a salt in DNA isolation, cold treatment should be avoided. The LiCl treatment in our protocol removed a major junk of RNA (Fig.1). The reason for early treatment with LiCl is the fact that LiCl is shown to be inhibitory to DNA replication in *in vivo* systems (Anwander et al., 1990). To avoid such disadvantages

the subsequent treatments may lessen the amount of any residual LiCl. LiCl is more powerful than NaCl in salting out the polysaccharides. Addition of BSA has manyfold benefits. It is known that BSA is a polyphenol absorbent (Couch and Fritz, 1990) and it prevents denaturation of proteins. In addition, BSA is useful as it binds a wide range of inhibitory complexes that are carried over during the DNA extraction proce-

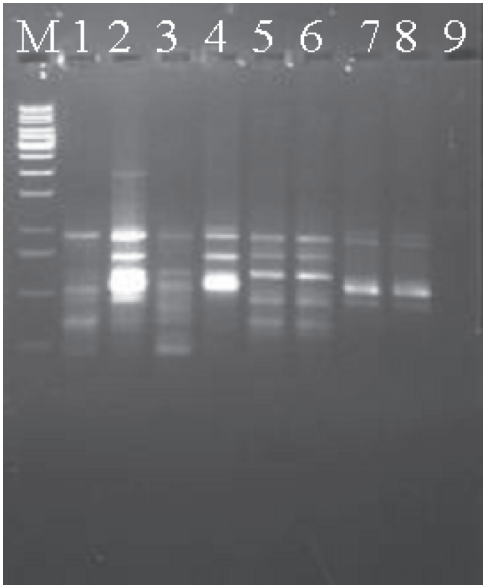

Fig 2. PCR profiles of DNA samples amplified with RAPD primer PL-136:5 /-GGAGTACTGG-3 / of four species (young and old leaves, respectively). 'M' represents 1 Kb DNA marker, Lane 1 and 3: *Withania somnifera,* Lane 2 and 4: *Tinospora cordifolia,* Lane 5 and 6: *Pueraria tuberosa,* Lane 7 and 8: *Cymbopogon pendulus,* Lane 9: negative control.

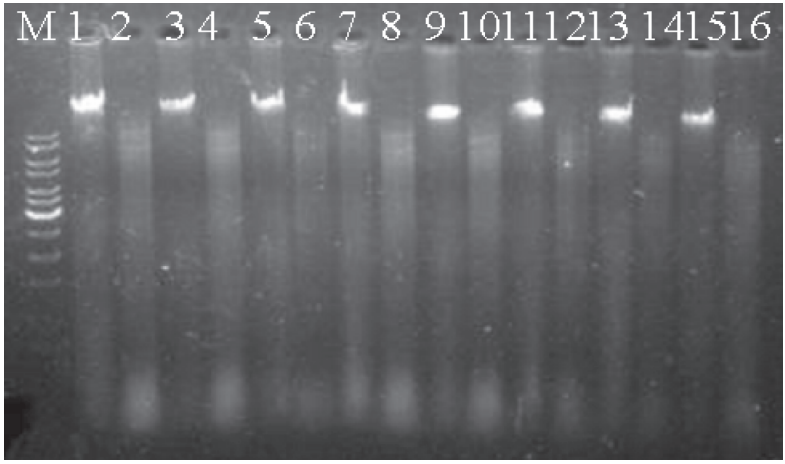

Fig 3. Restriction digestion of DNA isolated from four species (young and old leaves, respectively) with the restriction endonuclease *Hind* III along with their controls (nondigested and digested, respectively). 'M' represents 1Kb DNA marker, Lane 1-4: *Withania somnifera,* Lane 5-8: *Tinospora cordifolia,* Lane 9-12: *Pueraria tuberosa,* Lane 13-16: *Cymbopogon pendulus.*

dure (Sul and Korban, 1996). BSA is also an enhancer of DNA amplification (Kreader 1996), restriction and ligation (Sambrook et al., 1989). Thus, BSA can counter any inhibitory effect of LiCl in DNA amplification. In this respect, addition of BSA in the DNA extraction buffer is more advantageous than polyvinylpyrolidone (PVP-40). We tested several BSA concentrations within the range 0.02-0.2% and found the results satisfactory, except that at higher concentrations the residual BSA hindered the electrophoretic movement of DNA.

Polysaccharide contamination in isolated DNA inhibits enzymatic reactions, such as *Taq* DNA polymerase amplifications (Pandey et al., 1996) and restriction endonuclease cleavage (Raina and Chandlee 1996; Abdulova et al., 2002). However, in the present study, successful amplification (Fig. 2) and restriction digestion (Fig. 3) indicates the purity of DNA obtained by our method. We obtained DNA amplification using RAPD primers in all tested samples (Fig. 2). Similar to the restriction digestion (Fig. 3) with restriction endonuceases (data shown only for *Hind* III), amplification of DNA was complete, thus indicating unequivocally the suitability of DNA not only for Southern blotting and AFLP, but also for cloning purposes. We also used like Khanuja et al. (1998) DNA with and without phenol-chloroform treatment and found no differences in its potentiality for PCR or restriction digestion. Using the present protocol we obtained high quality DNA with fairly good amount even in older leaves, which are otherwise thought to be recalcitrant for DNA isolation. This protocol can probably be extended also to other angiosperm species.

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