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A simplified high yielding miniprep genomic DNA extraction protocol for three chemotypically different plant species

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The presence of metabolites has been observed to create interference with DNA isolation procedures and downstream reactions, such as, DNA amplification, restriction and cloning. Moreover, the chemotypic heterogeneity among species may cause hindrance in optimal DNA yields with a single protocol. Thus, even closely related species may require different isolation protocols. A rapid and high yielding DNA extraction procedure from heterogeneous plant tissues of 3 chemotypic different plants has been presented here. This modified method required grinding of plant tissues in DNA extraction buffer [200 m*M* Tris-HCL (*p*H 8.0), 200 m*M* NaCl and 25 m*M* EDTA, and 1% PVP], followed by phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) treatment. Ethanol in the presence of high salt concentration was then used to precipitate the DNA and for removal of polysaccharides. The average yield of DNA ranged from 1.68 to 5.37 mg g^{-1} of plant tissue and the purity was 1.6 to 1.9. The DNA was quite suitable for PCR using RAPD and also for restriction digestion. This method did not require liquid nitrogen for fixation, RNase treatment or storage at –80°C, making it advantageous over other common protocols.

Keywords: DNA extraction, PCR, phenol-chloroform, restriction digestion, SDS

The emergence of molecular marker analysis in genome studies has greatly enhanced the speed and efficiency of crop improvement and breeding programmes, which require the genotyping of a large number of individuals for diversity analysis, marker-assisted selection and variety fingerprinting. For high-throughput genotyping programme, the DNA must be of sufficient quality to generate robust and easily scored data with minimal repeats. In a genotyping programme, to carry out large amount of mini preparations is a very nerve wracking affair. Consequently, the method should be fast and simple. Since the DNA extraction method first cited by Marmur[1] in 1961 using SDS and chloroform, scientists have been devoting themselves to the improvement and simplification of DNA extraction methods. Most improved or simplified DNA extraction protocols require large amounts of starting materials to be ground in liquid nitrogen and costly chemicals or equipments that subject the total nucleic acids to a mixture of RNases and glycoside hydrolases, which degrade RNA and polysaccharides without affecting DNA integrity. There are many DNA extraction protocols established for different plant species, as different plant contain different amounts of nucleic acids and the chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation strategies[2,3]. The problem arises in downstream application because of many PCR-inhibiting substances, such as, tannins, polysaccharides, pigments and the ubiquitous DNA binding polypeptides, that can lead to poor PCR amplification and are unsuitable for RFLP (restriction fragment length polymorphism) and AFLP (amplified fragment length polymorphism) analysis[4,5]. Several methods have been used to overcome these problems including sedimentation in cesium chloride gradients or extraction with CTAB, selectively binding the DNA by using a RPC-5 column or by selective binding to an ion exchanger to avoid their co-precipitation with DNA after alcohol addition. The efforts demanded and the times required are almost equivalent in every case. The isolation of good quality DNA from plants like *Trichosanthes* sp., *Cicer* sp. and *Bacopa* sp. is complicated due to the presence of phenolic compounds, highly viscous polysaccharides, glycosides, flavonoids and DNA degrading endonucleases.

Considering the above issues, authors have made substantial modifications to make the SDS-based DNA extraction protocol[6] more reliable, rapid and economical. This modified protocol could also use small samples of plant tissue from plants of critical importance in the semiarid tropics. Moreover, the

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resulting DNA would be of excellent quality, suitable for molecular analysis with RFLP, AFLP and other PCR-based markers.

Leaves of field grown pointed-gourd (*Trichosanthes dioica* Roxb.), callus of chickpea (*Cicer arietinum* L.) and leaves of *in vitro* grown Bacopa (*Bacopa monnieri* L.) were harvested fresh before DNA isolation. About 100 mg of plant material was grounded with mortar and pestle in the presence of 1 mL of extraction buffer [200 m*M* Tris-HCL (*p*H 8.0), 200 m*M* NaCl and 25 m*M* EDTA, and 1% PVP]. The homogenate was transferred to 1.5 mL microcentrifuge tube and vortexed for 30 sec after adding 50 µL of 10% SDS solution. The tube was kept in a water bath at 65°C for 20-40 min and then centrifuged at 17000 rpm for 10 min, which separated the homogenate into two parts. The upper aqueous phase was taken into a new tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was mixed and again centrifuged at 12,000 rpm for 10 min. The supernatant was removed, and to which 1/5th volume 2 *M* NaCl and 2 volume prechilled ethanol was added and kept in –20ºC for 10 min to precipitate DNA. The DNA was spooled and transferred to another tube. The spooled DNA was washed with 70% ethanol (2-3 times) and air dried under laminar flow chamber. The DNA was dissolved in sterile double distilled water. The isolated DNA was quantified using spectrophotometer between 220 and 320 nm. The amount of the DNA present in the solution was calculated from absorption at 260 nm (A_{260}) and the purity of DNA was calculated by A_{260}/A_{280}. The quality of the extracted DNA was tested by running the DNA in 0.8% agarose gel and examined under UV transilluminator. The recorded mean data of isolated DNA (quantity and quality) were analyzed statistically.

DNA amplification was done using arbitrary decamer primers (Bangalore Genei, India) adopting the standard procedure[7]. PCR reactions were carried out in a 25 µL reaction mix containing ~30 ng template DNA, 200 µ*M* of dNTPs, 5 *p*mol of a single 10-mer primer, 0.2 unit of *Taq* DNA polymerase (Bangalore Genei, India), 1.5 m*M* $MgCl_2$ and 2.5 µL of 10× assay buffer. PCR reactions were performed in a thermocycler with a heated lid (Gene Amp PCR system 2400, Perkin Elmer). PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide (0.4 µg/mL) and UV illumination. For restriction analysis, the DNA was restricted by *Eco*RI (Bangalore Genei, India) using 3 U/µg 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.

Following the present protocol, good quality, intact DNA of high mol wt was obtained in larger quantities from the 3 chemotypically different species employed in the study (Fig. 1). The A_{260}/A_{280} ratio of the isolated DNA ranged from 1.70-1.82 (Table 1), while the A_{260}/A_{230} ratio was greater than 2 (data not shown). The DNA yield ranged from 1.80-5.30 µg/µL; thus, achieving a sufficient amount of nucleic acid from the small quantities of tissues (100 mg). Nevertheless, the quantity of isolated DNA

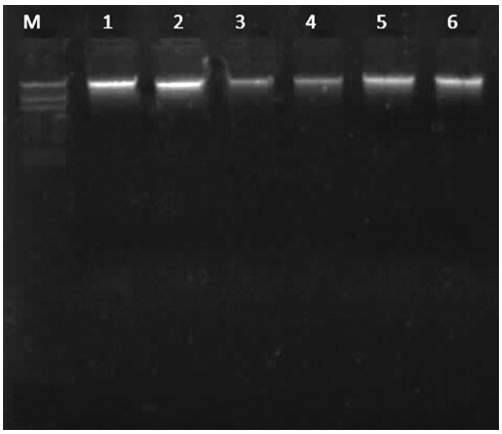

Fig. 1—DNA isolated from three plant species (M: Marker, λ DNA digested with *Hind*III, lanes 1 & 2: *C. arietinum*, lanes 3 & 4: *T. dioica*, lanes 5 & 6, *B. monnieri*)

Table 1—Quantity and purity index of extracted DNA from three plant species

No.	Sample	A_{260}	A_{260}/A_{280}	DNA quantity (µg µL^{-1})
1	*Bacopa monnieri* (L.) Penn.	0.179±0.04	1.72±0.00	2.63±0.17
2	*Cicer arietinum* L.	0.317±0.03	1.82±0.03	5.30±0.14
3	*Trichosanthes dioica* Roxb.	0.124±0.01	1.70±0.03	1.80±0.08

DNA diluted to three hundred times
Results are expressed as mean of three extractions with standard errors (SE)

has been dependent on the species, the genome size, the ploidy, the size of the cells at different phenological phases of the plant, the amount and type of tissue, the proportion between high quantities of tissue and volume of the extraction buffer used, and the effect of some substances in the extraction buffers that maintain the stability of the pure DNA[8]. In the present study, the maximum yield was achieved from the callus tissues of chickpea as compared to the tissue samples of pointed-gourd and Bacopa. The concentration of chemicals in the solution and the order of the addition of the chemicals could be vital, as it has been reported that adding SDS after 1 h of incubation in extracting buffer, instead of adding it directly to the buffer, could improve the efficiency[9]. In the present study, the traditional protocol[6] was modified by using SDS as a detergent with NaCl for solubilization of cell membrane and release of the cell contents. PVP was also used in the extraction buffer to remove polyphenols, which in their oxidized state can bind to the DNA and protein, making it brown in colour and useless for any research application. Phenol:chloroform:isoamyl alcohol (25:24:1) was used to remove the colouring substances like pigments and dyes. Subsequent extraction with chloroform ensured the removal of any lingering traces of phenol from the nucleic acid preparation. Ethanol with high concentration of NaCl was used for the precipitation of DNA and also for the removal of polysaccharides. DNA precipitation in the presence of ethanol often yields a large gelatinous mass of unknown composition. DNA is clearly visible within the mass, but difficult to separate from it. Simplest solution to the problem has been to dilute the aqueous phase obtained after chloroform extraction or to increase the NaCl concentration prior to alcohol precipitation. A high-salt buffer (1.5-2.0 *M* NaCl) proved effective for the isolation of genomic DNA from muskmelon, cucumber, potato, soybean, and geranium[10]. At this level, the polysaccharides remain in solution and are discarded with the ethanol supernatant, decreasing the levels of polysaccharide in the precipitated DNA. The use of NaCl in high concentration may perhaps also neutralize the acidic polysaccharides, which otherwise interfere with the activity of *Hind*III enzyme or *Taq* DNA polymerase in downstream applications[11]. In the present study, DNA was spooled, which facilitated the removal of excess amount of sodium salt and other contaminants. DNA pellet was finally washed with 70% ethanol, in which SDS remains in soluble state.

The purity and clean nature of DNA samples could be confirmed through complete digestion by the restriction enzyme *Eco*RI (3U/µg DNA; Fig. 2). This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA fingerprinting. The good utility of the isolated DNA in PCR amplification for RAPD profiling was demonstrated with RAPD primer (5'GCACGCCGGA3') and with preparations of all the plant species tested (Fig. 3).

In the present study, most of the concerns have been addressed in miniprep DNA extraction protocol for plants rich in secondary metabolites or polysaccharide derivatives. The present method required small amount of tissues for successful extraction and large numbers of samples could be processed in parallel. The method performed with phenol/chloroform to purify DNA rather than more expensive cesium chloride or CTAB used in other methods. It needed about 1 h to prepare DNA for any molecular biology application and there was no need of liquid nitrogen during crushing the plant material. This method also bypassed RNase treatment for DNA

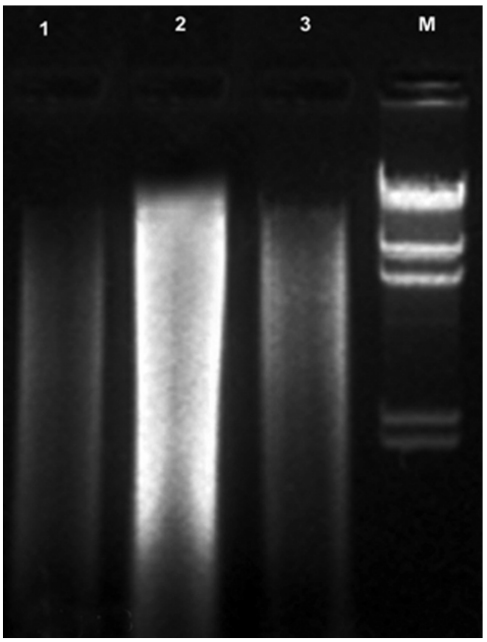

Fig. 2—Restriction digestion of DNA isolated from three plant species (Lane 1: *T. dioica,* lane 2: *C. arietinum,* lane 3: *B. monnieri,* M: Marker, λ DNA digested with *Hind*III)

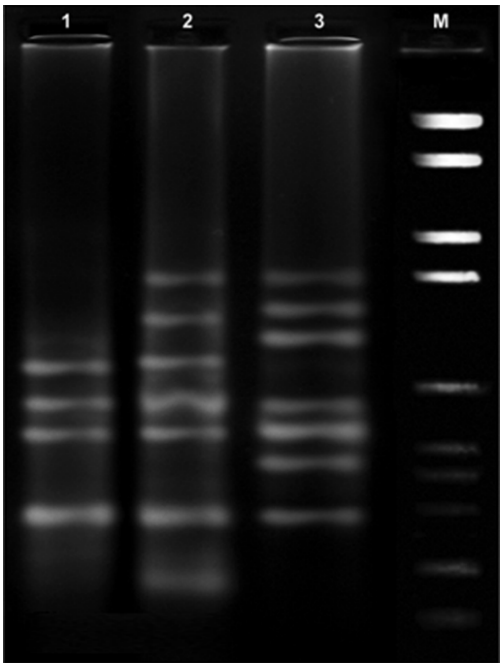

Fig. 3—PCR profiles of the DNA samples amplified with decamer primer MS10A (5' GCACGCCGGA 3') (Lane 1: *B. monnieri,* lane 2: *C. arietinum,* lane 3: *T. dioica ,* M: Marker 100 bp).

purification. The present protocol could be employed for miniprep as well as maxiprep DNA isolations. The protocol was efficiently employed in heterogenous plant materials both in terms of origin and identity. This method did not require expensive and highly hazardous reagents. It could be performed even in low technology laboratories. The quantity and the quality of the DNA extracted by this method were high enough to perform thousands of PCR-based reactions and could also be used in other DNA manipulation techniques. The protocol could be further extended to related species with relatively minor modifications. 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. Above all, a high level of genomic DNA with fair quality suitable for PCR-based markers and other genomic studies has successfully been extracted.

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