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A modified method to isolate genomic DNA from plants without liquid nitrogen

With the development of various molecular markers based on PCR, like RAPDs, SSRs, STRs, AFLP and PCR–RFLP, molecular biology has greatly enhanced the speed and efficiency of crop improvement and breeding programmes, rDNA technology and genomic DNA library construction. A pre-requisite for applying these methods is the ability to isolate high-quality genomic DNA of adequate quantity. A good extraction procedure is considered to be one which results in DNA of reasonable purity without the use of harmful chemicals. DNA extraction has been reported from various plant species using the cetyltrimethyl ammonium bromide (CTAB) procedure[1] and its modifications[2,3]. Most extraction methods employ expensive and hazardous procedures of grinding plant tissue in liquid nitrogen (N_2) to break down the cell wall of plants[4,5] or freeze-drying (lyophilization)[6]. Procurement and storage of liquid nitrogen may be difficult for many laboratories and handling of the same is also cumbersome. Thus any method not using liquid nitrogen is helpful. The omission of a grinding step in liquid nitrogen has been applied by many workers mostly using soft tissues such as flower petals[7] or young leaves[8,9]. In the present study a quick, simple and cheap procedure to isolate DNA from plants has been developed, which involves alternate cold (–80°C) and heat shock (60°C) treatments in order to break down the cell wall without using liquid N_2, which is suitable for various molecular biology applications. The extracted DNA was successfully subjected to PCR amplification of the ITS (internal transcribed spacer) region of rRNA gene, restriction digestion of the amplified product, microsatellite fingerprinting and RAPD successfully.

Samples of young, tender, leaves of ten plants species (*Desmodium giganicum, Aegle marmelos, Solanum xanthocarpum, Solanum indicum, Tribulus terresteris, Oroxylum indicum, Boerhavia diffusa, Trianthema portulacastrum, Trianthema monogyna* and *Datura innoxia*) were collected from the Botanical Garden of Panjab University, Chandigarh and various other nurseries. The plant tissue was washed well with water and sterilized by wiping it with 70% alcohol. The fresh weight of the plant tissue was taken and then it was chopped into fine pieces and subjected to DNA isolation. Simultaneously leaves of the same species were dried at 60°C and also processed for extraction of DNA.

DNA was extracted by the following steps:

- Pre-chill the mortar–pestle at –80°C for 15 min prior to the start of the experiment. Alternatively, it can be pre-chilled at –20°C for 1 h.
- Transfer the finely chopped plant tissue (300 mg) and dried tissue separately to chilled mortar–pestle and keep it at –80°C for 20 min or at –20 for 1 h.
- Grind the plant tissue into fine powder, transfer it to 1.5 ml microfuge tube and incubate it at 60°C for 5 min.
- Keep it again at –80°C for 15 min or at –20 for 1 h.
- Finally, thaw the powdered tissue by pouring equal volume (300 µl) of hot (65°C) 2× CTAB buffer (100 mM Tris [pH 8], 20 mM EDTA [pH 8], 1.4 M NaCl, 2% CTAB w/v, 2% PVP 40,000) and 1/10th volume of β-mercaptoethanol, and mix well.
- Add one volume (600 µl) of chloroform/isoamyl alcohol (24 : 1) and mix thoroughly to form an emulsion.
- Centrifuge in a microfuge for 15 min at 12,000 rpm.

- Transfer the supernatant solution from the top (aqueous) phase into a new microfuge tube. Discard the lower (chloroform) phase.
- Add 1/5th volume of 5% CTAB solution (5% CTAB w/v, 0.7 M NaCl) and mix well.
- Perform another chloroform/isoamyl extraction as mentioned above.
- Add equal volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris [pH 8], 10 mM EDTA [pH 8]) and mix gently. Place on ice for 30–60 min (At this step it can be kept at –20°C overnight).
- Centrifuge for 15 min. Discard the supernatant.
- Rehydrate the pellet in high-salt TE buffer (10 mM Tris [pH 8], 1 mM EDTA [pH 8], 1 M NaCl).
- Add two volumes of cold absolute ethanol or isopropanol and mix gently by inverting the tube 8–10 times.
- Centrifuge for 15 min. Discard the supernatant.
- Wash the pellet with 70% ethanol. Invert the tubes on tissue paper or paper towel to drain out the liquid.
- Dry the pellet for 20–30 min or until all the liquid is evaporated.
- Rehydrate the pellet in 100 µl 0.1 × TE buffer (1 mM Tris [pH 8], 0.1 mM EDTA [pH 8])
- Treat with RNAase (25 µg/ml) for 3 h at 37°C.
- Perform another chloroform/isoamyl extraction as mentioned above.
- Add two volumes of cold absolute ethanol or isopropanol and mix gently by inverting the tube 8–10 times.
- Centrifuge for 15 min. Discard the supernatant.
- Wash the pellet with 70% ethanol. Invert the tubes on tissue paper or paper towel to drain out the liquid.
- Dry the pellet and dissolve in 100 µl 0.1× TE buffer.
- Store at –20°C.

The yield of DNA per gram of leaf tissue was quantitated using UV–VIS spectrophotometer by reading absorbance at 260 nm, purity was checked by taking the ratio at 260/280 nm.

The ITS region comprising ITS1–5.8S rRNA gene–ITS2 of isolated DNA from these plants was amplified using primer sets ITS1F (5′-AAG TCG TAA CAA GGT TTC CGT AG-3′) and ITSR (5′-TCC TCC GCT TAT TGA TAT GC-3′). Amplification reaction was performed using Eppendorf Master Cycler following White et al.[10]. Amplified products were resolved in 1.2% agarose gel in 1 × TAE buffer for 1 h at 75 V. A 100 bp DNA ladder served as the standard molecular weight marker. The gel was stained with ethidium bromide and documented under gel documentation system.

The restriction enzyme digestion was carried out using 1 µg of amplified PCR product digested with 10 units of restriction enzyme for 16 h. The enzymes used for were BamH1, HindIII, MspI, MboI, EcoRI, EcoRV, HinfI, AluI, according to the specifications of the manufacturer. After inactivation of enzymes, the restriction fragments were size separated by electrophoresis on 2% agarose gel at 60 V for 2 h. A 100 bp DNA ladder served as the standard molecular weight marker. The staining and documentation were the same as in ITS amplification.

For a 50 µl mixture, each tube consisted of 2.0 µl (0.1 µg) template DNA, 5.0 µl of 10 × Taq buffer with 15 mM magnesium chloride (1 ×), 1.0 µl of 25 mM magnesium chloride (0.5 mM), 5.0 µl (0.2 mM each) of dNTPs (2 mM), 1.0 µl (20 pmol) of primers $(GTG)_5$, $(GAC)_5$ and $(GACA)_4$ separately, following Baleiras Couto et al.[11]. After the completion of the cycles, the sample was tested for amplification as described previously.

High molecular weight DNA was obtained from all the samples reported here (Figure 1). In experiments where DNA isolation was carried out using this method, the plant samples were incubated at –80°C for different time periods, i.e. 5, 10, 15, 20, 25, 30, 60 and 90 min. The best DNA yield was obtained when the incubation was carried out for 20–30 min or more. Pre-chilled mortar–pestle helped save time in bringing the temperature of the tissues to –80°C. DNA yield obtained using this method varied between 40 and 340 µg/ml per 150 mg of fresh leaf material (Table 1). Yield variation depended upon the maturity of the tissue samples. Young leaves gave high DNA yield, with less or
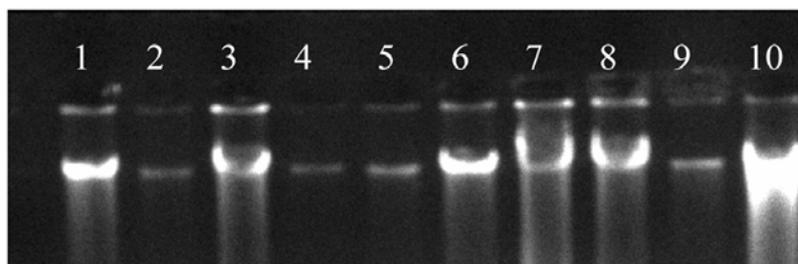
Figure 1. DNA extracted from the leaves of various plants. Lanes 1–10, DNA isolated from *Aegle marmelos*, *Desmodium giganticum*, *Gmelina arborea*, *Oroxylum indicum*, *Solanum indicum*, *Solanum xanthocarpum*, *Tribulus terrestris*, *Trianthema monogyna*, *Trianthema portulacastrum* and *Boerhavia diffusa* respectively.

Table 1. Concentration and λ260/λ280 ratio of extracted DNA from fresh leaves of different plants

Plant	DNA concentration (µg/ml)	Ratio (λ260/λ280)
Boerhavia diffusa	7.20	0.96
B. diffusa (Chandigarh)	82.08	2.36
B. diffusa (Gujarat)	17.60	0.66
Aegle marmelos	42.18	5.96
Trianthema portulacastrum (Chandigarh)	40.62	2.51
T. portulacastrum (Amritsar)	18.05	0.82
Gmelina arborea	425.16	1.55
Trianthema monogyna (Chandigarh)	421.92	1.56
T. monogyna (Chandigarh)	334.86	1.70
Premna integrifolia	15.84	1.30
Tribulus terrestris	20.58	1.80
Solanum xanthocarpum	249.38	1.60
Solanum indicum	43.44	1.60
Oroxylum indicum	30.00	2.50
Desmodium giganticum	150.25	1.70

no contamination of protein or carbohydrate. The yield was sufficiently good, 40–220 µg/ml per 300 mg of dried tissue. The $\lambda 260/\lambda 280$ ratio was greater than 1.8 (ranging from 1.55 to 2.51), showing the presence of protein and polysaccharide contamination. If the sample contained high amounts of polysaccharides or other metabolites, the quantity of DNA obtained was also reduced. For such plants, the time period for –80°C incubation was increased by 15–30 min. Certain antioxidants, e.g. β-mercaptoethanol and PVP helped remove the polyphenolic content and yielded more pure DNA (Figure 1). Increasing the time period of 60°C incubation from 2 to 4 min between the two –80°C incubation did not result in any significant change in DNA yield.

DNA extracted using this method was used for PCR amplification of the ITS region. The ITS regions of ten samples were successfully amplified and ranged between 680 and 850 bp (data not given).

When the amplified ITS product was digested with eight enzymes separately, *Bam*HI and *Hin*dIII had restriction sites only in the ITS of *Desmodium giganticum* and *Solanum indicum* respectively. The rest of the enzymes gave restriction fragments in all the plant species, except *Eco*RI which did not have sites for any of the ITS products (data not given).

Microsatellite fingerprinting showed uniform banding with all the three primers in some species and few variations in some, indicating intra-specific variations in species studied (data not given).

An alternate cold (–80°C) and heat (60°C) shock treatment was given to both fresh and dry tissues in order to break down the cell wall, instead of using liquid N_2. The DNA extracted by this method is suitable for PCR amplification of genes, PCR–RFLP, microsatellite fingerprinting and RAPD (data not shown), and gave a consistent result for all the plants used. It is also suitable for both dicotyledonous and monocotyledonous plants. In most of the earlier reports of DNA extraction without liquid nitrogen, fresh and young tissue or callus was used[8,9,12]. We have successfully extracted DNA with sufficient yield from both fresh as well as dry tissues. Since only 10–20 ng DNA is required for PCR amplification, several 100 reactions can be set up with such a yield. Another added advantage of this method is the use of –20°C freezer in case of non-availability of –80°C freezer. This would however require more time for extraction.

In conclusion, this method can be used for extraction of DNA of high-quality and high yield from any type of plant tissue and is suitable for all types of molecular biology experiments. The extracted DNA was stable and gave the same results in PCR, microsatellite fingerprinting, PCR–RFLP and RAPD after 2 years of storage at 4°C.

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Corolla elongation as an aid in self-pollination in *Rhamphicarpa longiflora* (Scrophulariaceae)

Rhamphicarpa Benth. is a small, Old World genus with only six species[1]. Like other members of the tribe Buchnereae of Scrophulariaceae, the genus *Rhamphicarpa* includes species that are root parasites on a wide range of hosts.

Rhamphicarpa longiflora Wight ex Benth. is the only species found in the Indian subcontinent restricted to peninsular India. It is a component species of herbaceous monsoon vegetation found on soils of rocky plateaus ranging in altitude from 30 to 1200 m, or on marshy grounds. According to Cisse *et al.*[2], moth pollination is known only in the genus *Cycnium* and *Rhamphicarpa* in the parasitic Scrophulariaceae, but very little is known about pollination in *R. longiflora*. We studied the pollination ecology of *R. longiflora* to answer the following questions: (1) What is the phenology? (2) Are moths the sole legitimate pollinators? (3) Is the species self-pollinated or cross-pollinated? (4) Does corolla play any role in self-pollination? (5) Why does the species adopt self-pollination?

Studies on pollination biology of the species were made during rainy months from August to October in 2007 and 2008. Flowering and fruiting periods