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An Optimised Protocol for Fast Genomic DNA Isolation from High Secondary Metabolites and Gum Containing Plants

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Abstract: Extraction of high quality DNA from plants like *Commiphora wightii* containing secondary metabolites, polysaccharides and phenolic compounds often becomes challenging as many of these compounds co-precipitate with DNA and make the DNA unsuitable for downstream applications. Previously reported protocols yielded highly viscous and slimy DNA preparations that were not amenable to further analysis. We circumvented this by modifying the composition of the homogenisation buffer, by increasing the amount of cetyl trimethylammonium bromide (CTAB), polyvinyl pyrrolidone (PVP) and β -mercaptoethanol; combining RNase A treatment along with the lysis step and precipitating DNA using isopropanol at room temperature. The DNA isolated using this optimised protocol was high in quality and suitable for restriction endonuclease digestion as well as polymerase chain reaction (PCR) mediated amplification for applications in biodiversity studies.

Key words: *Commiphora wightii*, DNA extraction, CTAB-PVP, restriction endonucleases, PCR

INTRODUCTION

The isolation of superior quality, high molecular mass genomic DNA, essential for several molecular biology applications, becomes difficult for many medicinally important plants due to the presence of secondary compounds. These compounds interfere and inhibit PCR, restriction digestion and other downstream experiments and make the application of these procedures limited for further genetic analysis and improvement of these valuable plants. *Commiphora* spp. of the family Burseraceae are found in the arid to semi-arid regions of the world including the deserts of Africa, Saudi Arabia, India and Pakistan. The plant has been featured in the IUCN red data book as Data Deficient and vulnerable (IUCN, 2008) and immediate strategies for evaluation of available germplasm had been suggested for biodiversity conservation (Khan *et al.*, 2003). The plants contain a bitter gum known as guggul (Myrrh) in stems and leaves. The yellowish gum oozes upon making an incision and solidifies in the hot environment to a hard brownish resin. Guggul is medicinally important and is used in the treatment of hypercholesterolemia and cardiovascular diseases (Singh *et al.*, 1994; Deng, 2007), it is also shown to have anti-cancerous activity (Xiao and Singh, 2008). The major components of gum guggul are conjugates of terpenes, lactones, steroids that are produced

constitutively as secondary metabolites (El Ashry *et al.*, 2003) for defence against pests (Becerra, 1997). Fructans, polysaccharides and phenolics are also present in huge quantities in the plant.

CTAB based DNA isolation and purification procedures (Doyle and Doyle, 1990) have been successfully applied to a number of plant species having secondary metabolites like blackcurrant (Woodhead *et al.*, 1998), conifers (Kim *et al.*, 1997) and ferns (Dempster *et al.*, 1999). Highly viscous slime like non-amplifiable DNA was obtained from *Commiphora* spp. leaves following the conventional procedure. The presence of residual polyphenolics and polysaccharides makes the procedure problematic and renders the DNA unsuitable for digestion by restriction enzymes and amplification by *Taq* DNA polymerase (Porebski *et al.*, 1997; Michiels *et al.*, 2003). Modifications of the original procedure were reported for isolation of DNA from plants with high polysaccharides and polyphenolic compounds (Saghai-Marooof *et al.*, 1984; Rether *et al.*, 1993; Tel-Zur *et al.*, 1999; Michiels *et al.*, 2003; Dehestani and Kazemi Tabar, 2007). However none of these previously published protocols were found appropriate for extracting high quality DNA from the gum containing *Commiphora* spp. The aim of this study was to establish a simplified protocol for isolation of high quality DNA from *Commiphora* spp. for the generation of molecular markers for biodiversity analysis.

MATERIALS AND METHODS

Plant material: *Commiphora wightii* an endangered, medicinally important, gum containing plant was used for the isolation of DNA. Leaves appear on the plants only during the short rainy season (July-October). Samples were collected for the present study in August 2005. Twigs with 3-5 leaves were collected from plants growing naturally in the hilly tracts of the Thar Desert in Rajasthan, India, kept in zip lock bags in icebox for transportation to the laboratory at Jaipur, Rajasthan and DNA was isolated the following day.

Preparation of homogenisation buffer: The homogenisation buffer consisted of 150 mM Tris. HCl (pH 8.0), 1.5 M NaCl and 25 mM EDTA (pH 8.0). The buffer was autoclaved and stored at room temperature (24°C). Just before use, pre-quantified volume of the buffer was warmed to 65°C in autoclaved bottles. CTAB, 3.5% w/v (Bangalore Genei, India) and PVP, 3.0% w/v (Calbiochem) were added and allowed to dissolve by gentle intermittent swirling. Prior to homogenisation β -mercaptoethanol (0.3% v/v) was added.

DNA Extraction procedure: Leaves (0.4 g) were de-veined and grinded to a fine powder in a mortar and pestle using liquid nitrogen. The powder was suspended in 3 mL of pre-warmed, completely prepared homogenisation buffer to get a suspension. The suspension (750 μ L) was distributed in 1.5 mL sterile eppendorf tubes using wide bore pipette tips. Five microlitres of RNase A (stock 20 mg mL⁻¹) was added to each tube and incubated for 45 min at 65°C with occasional gentle inversions.

The tubes were allowed to cool to room temperature and to each tube equal volume of chloroform: isoamyl alcohol (24:1) was added. The tubes were inverted gently 20-25 times to form an emulsion and were centrifuged at 13000 g for 10 min at room temperature in a microfuge (Biofuge, Heraeus).

The upper aqueous layer was carefully pipetted out into fresh eppendorf tubes without disturbing the inter-phase using wide bore pipette tips. Isopropyl alcohol (0.6 volumes) was added to the transferred aqueous phase, mixed well and incubated at room temperature for 30 min.

The tubes were centrifuged at 13000 g for 15 min at room temperature and the supernatant was gently poured off. The pellets were washed thoroughly with 750 μ L of 70% ethanol and were again spun as before, but for only 5 min. The supernatant was discarded and the pellets were air-dried (~30 min). The pellets were allowed to re-suspend in 30 μ L of TE (10 mM Tris. HCl pH 8.0; 1 mM EDTA pH 8.0) at 4°C overnight.

The isolated DNA was subjected to electrophoresis on 0.8% agarose gels prepared in 1×TAE (40 mM Tris acetate, 1 mM EDTA) electrophoresis buffer to check the quality. Quantification of the isolated DNA was performed in a spectrophotometer (Biomate 3, Thermo Spectronic) at 260 nm and the purity was checked from A_{260}/A_{280} ratio. Isolated DNA was also quantified in a Fluorometer (VersaFluor, Biorad) fitted with excitation (360 nm) and emission (460 nm) filters using Hoechst 33258 (Sigma Aldrich) as the fluorochrome.

Restriction endonuclease digestion: One microgram of isolated genomic DNA was digested to completion with 10 Units of *Bam* HI, *Eco* RI, *Eco* RV, *Hind* III and *Xba* I (all from Bangalore Genei, India) for 16 h at 37°C using the recommended buffer in 50 μ L reaction volumes. DNA digestion was assayed by visual inspection on a UV transilluminator (GelDoc, 2000, Biorad) after 1% agarose gel electrophoresis and Ethidium bromide staining.

PCR amplification: DNA amplification reactions were assembled in 25 μ L volume consisting of 1×*Taq* polymerase buffer, 1.5 mM MgCl₂, 0.6 Units of *Taq* DNA polymerase, (all from Fermentas GmbH), 0.1 mM each of dATP, dCTP, dGTP and dTTP (Applied Biosystems), 0.5 μ M of random decamer (Operon Biotechnologies GmbH) and 30 ng of genomic DNA. Amplifications were performed using a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems) that was programmed for an initial denaturation step at 95°C for 4 min followed by 45 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min. In the final cycle, the elongation step at 72°C was extended by 5 min.

Internal Transcribed Spacers (ITS) region of nuclear ribosomal DNA (nrDNA) was also amplified by angiosperm ITS specific primers ITS5: 5'GCAAGTAAAAGTCGTAACAAGG 3' and ITS4: 5'TCCTCCGCTTATTGATATGC 3'. The Thermocycler was programmed for initial denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min with the final extension at 72°C for 10 min. Reaction mixture composition was same except 50 ng of template DNA, 0.25 μ M of each primer and 1.0 unit of *Taq* DNA polymerase was used.

The amplified products were size fractioned on 1.4% agarose gels and visualized as before.

RESULTS AND DISCUSSION

Isolation of genomic DNA ideal for a wide range of molecular biology applications like biodiversity studies is a fundamental requirement. We tried many commonly used protocols (Saghai-Maroo *et al.*, 1984; Doyle and

Table 1: DNA yield and purity as obtained for *C. wightii* using various published protocols

Published protocol	Homogenate incubation		RNase A treatment	CTAB (wtv %)	PVP (wtv %)	β -mercapto-ethanol (v/v %)	DNA yield ($\mu\text{g g}^{-1}$ leaf)	Purity A_{260}/A_{280}
	Time (min)	Temp ($^{\circ}\text{C}$)						
Saghai-Marooif	30-60	60	Not mentioned	1.0	Not used	0.1	89.76	1.62
Doyle and Doyle	30	60	Two step	2.0	Not used	0.2	92.12	1.67
Michiels	60	60	Two step	2.0	2.0	0.2	76.67	1.96
Optimized protocol	45	65	One step	3.5	3.0	0.3	70.02	1.91

Data are average of three replications

Doyle, 1990) but were unable to isolate high quality DNA from the gum containing plant *C. wightii* (Table 1). Published protocol with modifications (Michiels *et al.*, 2003) was also tried, but the DNA quality remained unsatisfactory. This may be attributed to the presence of polysaccharides, polyphenolics and other secondary metabolites as constituents of the gum in the leaves of *C. wightii*. To overcome this challenge, we had to optimise the contents of homogenisation buffer, omission of some extraction and washing steps and modification of DNA precipitation temperature and duration.

The optimized homogenisation buffer contained higher amount of CTAB and PVP as compared to the published procedures (Table 1). CTAB binds with polysaccharides and fructans (Gawel and Jarret, 1991), whereas PVP forms insoluble complexes with lactones and phenolics (Kim *et al.*, 1997). All these complexes are removed by precipitation during centrifugation after extraction with chloroform: isoamyl alcohol. Increasing the amount of β -mercaptoethanol from 0.2 to 0.3% helped to check the oxidation of phenolic compounds. Inclusion of phenol in the chloroform: isoamyl alcohol solution was avoided so as to get high average molecular weight DNA, as phenol might cleave phosphodiester bonds of DNA leading to its degradation. DNA isolated with this procedure was not viscous or slime like or gummy, indicating proper removal of contaminants. Addition of RNase A to the homogenate and incubation for 45 min at 65°C was found sufficient to degrade RNAs along with the lysis of cells as was evident after agarose gel electrophoresis (Fig. 1). Inclusion of RNase A during this initial incubation step not only lessens time for performing the entire procedure but also reduces labour. A separate RNase A treatment, as mentioned in many of the published protocols (Table 1) would also require additional once phenol: chloroform: isoamyl alcohol and once chloroform: isoamyl alcohol extraction and centrifugation steps to remove residual enzymes. This also eliminates the necessity of subsequent ammonium acetate addition and ethanol precipitation.

The temperature, incubation time and the type of alcohol used for precipitation of DNA also influenced its yield and quality (Table 2). Overnight precipitation at -20°C by both ethanol and isopropyl alcohol yielded

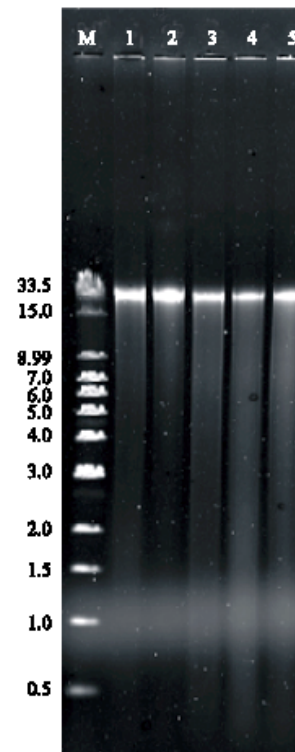


Fig. 1: Genomic DNA isolated from different plant species with high gum and secondary metabolite content. M: Molecular weight marker (DNA supermix, Bangalore Genei), Lanes 1 and 2: *Commiphora wightii*, Lane 3: *Ocimum tenuiflorum*, Lane 4: *Mimosa pudica*, Lane 5: *Shorea robusta*

Table 2: Effect of the type of alcohol used, incubation time and temperature on DNA yield and purity as obtained

Type of alcohol used	Incubation		Yield ($\mu\text{g g}^{-1}$ leaf)	A_{260}/A_{280}
	Time	Temperature		
Absolute ethanol	Overnight	-20°C	89.47	1.57
Absolute ethanol	Overnight	Room temperature	71.34	1.67
Absolute ethanol	30-60 min	-20°C	66.22	1.62
Absolute ethanol	30-60 min	Room temperature	55.47	1.96
Isopropyl alcohol	Overnight	-20°C	112.14	1.58
Isopropyl alcohol	Overnight	Room temperature	72.41	1.72
Isopropyl alcohol	30-60 min	-20°C	87.17	1.76
Isopropyl alcohol	30-60 min	Room temperature	70.02	1.91

Data are average of three replications

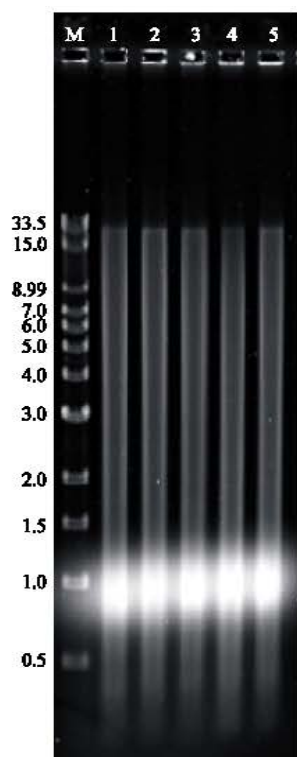


Fig. 2: Restriction endonuclease digestion pattern of Genomic DNA isolated using the optimised protocol from *C. wightii*. M: Molecular weight marker (DNA supermix, Bangalore Genei), Lane 1: *Bam* HI, Lane 2: *Eco* RI, Lane 3: *Eco* RV, Lane 4: *Hind* III, Lane 5: *Xba* I

higher amounts of DNA, but spectrophotometric observation indicated co-precipitation of contaminants (Table 2). Whereas precipitation at room temperature for shorter periods of time by both alcohols resulted in loss of yield but the recovered DNA was of superior quality (Table 2). Further it was noticed that the DNA pellet never completely dissolved in TE buffer when dried using a vacuum centrifuge. Hence air-drying by keeping the tubes containing the DNA pellet inverted on a piece of tissue paper was preferred.

The protocol optimised for *C. wightii* in our laboratory yielded DNA from 55-70 $\mu\text{g g}^{-1}$ of leaf tissue (Table 2) having A_{260}/A_{280} of 1.9 ± 0.1 and molecular weight of ~ 30 kb. Since densitometric measurements always are not that accurate for the detection of small amounts of DNA (Csai *et al.*, 1998), DNA isolated using the optimized protocol were also quantified in a fluorometer using Hoechst 33258 and compared with the spectrophotometer readings. Though the yield was less than published protocols by Saghai-Maroo *et al.* (1984)

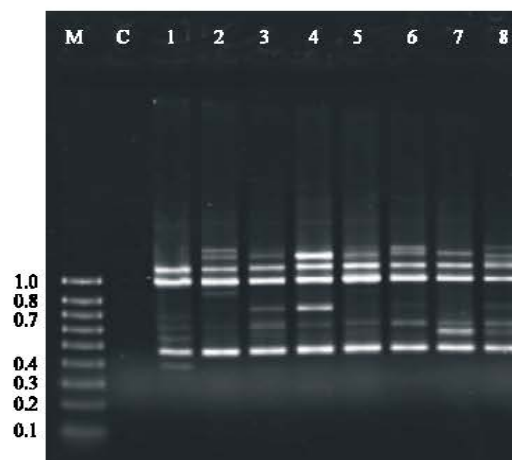


Fig. 3: RAPD profile of *C. wightii* generated by the primer OPA-11 (5'CAATCGCCGT3'). M: Molecular weight marker (Hyperladder IV, Bioline), Lanes C: Control-No template DNA, Lanes 1 to 8: Different biotypes of *C. wightii*

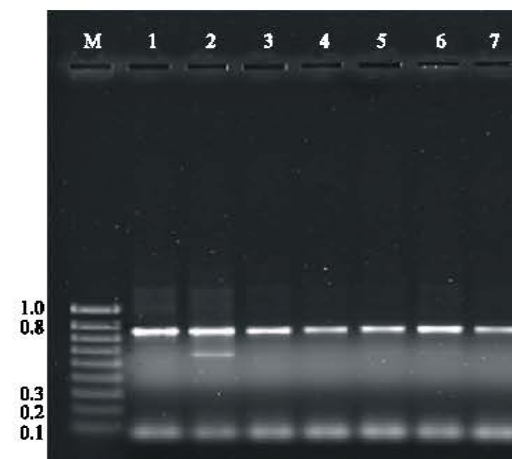


Fig. 4: PCR amplification of ITS regions of nrDNA of *C. wightii*, genomic DNA isolated by the optimised protocol using primers ITS-4 and ITS-5. M: Molecular weight marker (Hyperladder IV, Bioline), Lanes 1 to 7: Different biotypes of *C. wightii*

and Doyle and Doyle (1990) but the purity was much superior (Table 1). This was also evident upon visual observation of the agarose gels stained with Ethidium bromide after restriction endonuclease digestion (Fig. 2) and PCR amplifications using RAPD primers (Fig. 3) and ITS sequence specific primers (Fig. 4).

This easy-to-do, quick and inexpensive CTAB-PVP based procedure yielded sufficient DNA to be used as template for >800 amplification reactions. This optimised

procedure required only one transfer of the DNA solution, after the chloroform: isoamyl alcohol extraction step, thereby reducing the possibilities of DNA degradation as well as cross contamination. The entire procedure can be performed at room temperature eliminating the need for refrigerated centrifuge. Unlike all other protocols, this optimised protocol is relatively fast, taking less than 3 h and consistent producing very acceptable DNA yields from mature leaf tissue for the species assayed. All DNA preparations were found to be stable when stored at -20°C for more than two years and could be amplified by PCR using both random as well as targeted primers.

In conclusion, this technique has potential to be an effective protocol for DNA extraction using mature leaf tissue for *Commiphora* and other species in the family Burseraceae with high polysaccharide and gum content. Also, this protocol is routinely used in our lab to isolate DNA from a wide variety of plant samples including *Mimosa pudica*, *Ocimum tenuiflorum*, *Shorea robusta* all having secondary metabolites.

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