

Original Research Article

<https://doi.org/10.20546/ijcmas.2019.803.188>

A Simple and Efficient Genomic DNA Extraction Protocol for Dried Leaf of Threatened Species *Commiphora wightii* (Arnott) Bhandari for Genetic Analysis of Plant Biological System

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ABSTRACT

Commiphora wightii (Arnott) Bhandari contains secondary metabolites, polysaccharides and phenolic compounds. The presence of secondary metabolites, reduce the yield and quality of the DNA. In the present study an alternative protocol for genomic DNA extraction from dry plant leaves was developed that is acquiescent to PCR-based genomic studies. Existing protocols were lengthy, costly or not appropriate for genomic DNA extraction from dry leaves. This modified CTAB (3%) and PVP (Polyvinylpyrrolidone) 1.5% protocol include the use of 0.5M NaCl, 0.3% β -mercaptoethanol in the extraction as well as application of autoclaved sand for proper grinding of dried leaves and inclusion of RNase A treatment in the protocol to fasten the process. The extracted DNA using present optimized protocol was super in quality and quantity. It was also suitable for polymerase chain reaction with random decamer, inter simple sequence repeat and barcode primers. The developed protocol is rapid and cost efficient with high quality and sufficient quantity of DNA for downstream PCR-based genetic analysis.

Keywords

Commiphora wightii, Molecular markers, PCR amplification, polyvinyl pyrrolidone, β -mercaptoethanol, DNA extraction

Article Info

Accepted:
12 February 2019
Available Online:
10 March 2019

Introduction

Presence of secondary metabolites in plants interferes with extraction of good quality DNA for subsequent PCR based genetic analysis (Kotchoni *et al.*, 2011). DNA extraction protocols must be standardized for every plant species with higher level of these metabolites to simplify genetic analysis of plant biological system. Various expensive

DNA extraction protocols are available with lengthy procedure. Generally the available DNA extraction protocols recommend fresh leaf samples for isolation of genomic DNA, but it is unfeasible when the samples are collected from distant and rare places. These types of circumstances require the development of the protocols for extracting DNA from dried leaf samples. Guggul (*Commiphora wightii*) is a pharmacologically,

economically and ecologically important species that grows wild in state of Madhya Pradesh, Gujarat and Rajasthan in India. Generally guggul plants are found in arid and semi arid climate and shown tolerance to poor soil in Rajasthan. The plant contains essential oils, mainly myresene, dimyrecene and polymyrecene, Z-guggulosterone, E-guggulosterone. The presence of phytochemicals like steroid, saponins, tannins, flavanoids, and alkaloids has also been confirmed (Zaid *et al.*, 2015). Considering the above issues we have made substantial modifications to make the CTAB based DNA extraction protocol (Saghai-Marooof *et al.*, 1984) more reliable, fast and economical. This modified protocol is also able to give good yield with small samples of plant tissues. Moreover, obtained DNA would be of good quality suitable for molecular analysis.

Materials and Methods

Source of biological material

Leaf samples were obtained from *Commiphora wightii* plants from different locations of India (Table 1) and stored for genomic DNA isolation.

Chemicals, reagents and solutions

(i) DNA Extraction: 100mM Tris-HCl (pH 8.0), 20mM EDTA (pH8.0), 0.5M NaCl, 3% CTAB (Cetyl Trimethyl-Ammonium Bromide), 0.3% β -mercaptoethanol, 1.5% PVP (Polyvinylpyrrolidone), 24:1Chloroform-isoamyl alcohol (IAA), 3M sodium acetate (pH4.8), Isopropanol (4 °C), 70% ethanol.

(ii) PCR amplification and electrophoresis: 10X PCR buffer (1X working), 2.5 mM $MgCl_2$, 10mM dNTPs (200 μ M), 10pM Primer (RAPD and ISSR), *Taq* DNA Polymerase (5 Unit/ μ l), 50ng template DNA, Nuclease free H_2O for volume making, Agarose, 1X TAE, Ethidium bromide, primers, DNA ladder.

Preparation of DNA extraction buffer

The DNA extraction buffer was used for the homogenization of chemical 100 mM Tris (pH, 8.0), 0.5 M NaCl, 20 mM EDTA (8.0 pH). After adding 3% CTAB and 1.5% PVP the final volume was made up 100ml with nuclease free water. β -mercaptoethanol was added just prior to keeping DNA extraction buffer in water bath for incubation at 65°C.

Genomic DNA isolation

2g of fresh and healthy leaves were taken for genomic DNA isolation.

Leaf sample was homogenized in liquid nitrogen using a pestle and mortar and grind with liquid nitrogen in the presence of autoclaved sand to make fine powder.

The fine powder was transferred to 50 ml oakridge tube and 10 ml of DNA extraction buffer (preheated at 65°C) was added and mixed thoroughly. Sample tubes were incubated at 65°C in water bath for 1h, with intermittent mixing after 10 min during incubation.

10 μ l RNase A (20mg/ml) was added and mixed gently. Sample tubes were incubated at 37 °C for 40 min.

Equal volume of Chloroform: Isoamyl alcohol (24: 1) was added and mixed gently and tubes were centrifuged at 12,000 rpm for 12 min at room temperature. Supernatant was transferred to a fresh 50 ml oakridge tube and equal volume of chloroform: isoamyl-alcohol (24:1) was added again and mixed gently.

The mixture was centrifuged again at 10,000 rpm for 10 min at room temperature.

The supernatant was transferred to a fresh 50 ml tube and an equal volume of pre-chilled isopropanol was added and mixed gently by

inverting and kept for 10 min at room temperature without disturbing.

The precipitated DNA was then spool out using 1.2 ml cut tips and transferred to a 1.5 ml microcentrifuge tube.

DNA was pelleted by spinning at 10,000 rpm for 8 min. Supernatant was discarded and pellet was washed twice with 500 µl of 70% ethanol.

The pellet was dried up at room temperature and dissolved in 100µl Tris: EDTA buffer and stored at -20 °C for further use.

Testing of DNA quality and purity

Purity of DNA was checked by taking the ratio of Optical Density (O.D.) using Nanodrop-Spectrophotometer at 260nm to 280nm. The qualities of extracted DNA were tested by gel electrophoresis. It was done on 0.8% agarose gel stained with Ethidium Bromide samples and amplified fragments of DNA were observed in gel documentation system.

Amplification of DNA using primers

The PCR amplification procedure for amplification of DNA RAPD was followed as per protocol described by Williams et al (1990). The components and their concentration used in the RAPD and ISSR PCR reaction were prepared as follows: PCR amplification reactions volume 20µl consisting 2µl of PCR buffer, 2.4µl of MgCl₂, 0.2µl of *Taq* Polymerase (5 Unit/µl), 0.5µl of dNTPs, 2µl of Primer, 2µl of genomic DNA and nuclease free water to makeup the total volume. For DNA barcode primers (*rbcL* and *matK*) the components were used as follows: 1µl of PCR buffer, 0.7µl of MgCl₂, 0.1µl of *Taq* Polymerase (5 Unit/µl), 0.2µl of dNTPs, 0.5µl of forward primer, 0.5µl of reverse

primer, 1µl of genomic DNA and nuclease free water to makeup the total volume 10 µl. Amplifications were performed using “BIORAD T100 and Agilent Technologies Sure Cyclor 8800” programmable thermal cyclor with the cycling parameters that was programmed for ISSR an initial denaturation step at 94°C for 4 min followed by 45 cycles at 94°C for 45 second, 50°C for 1 min annealing and 72°C for 2 min elongation. In the final cycle, the elongation step at 72°C was extended by 5 min. Likewise; the temperature profile used in RAPD PCR amplification were the same except the annealing temperature was 37°C. The cycling parameters that was programmed for *rbcL* and *matK* primers were: an initial denaturation step at 94°C for 3 min followed by 35 cycles at 94°C for 30 second, annealing for *rbcL* at 55°C and for *matK* at 58°C for 30 sec and 72°C for 45 sec elongation. In the final cycle, the elongation step at 72°C was extended by 7 min.

Results and Discussion

Extraction of superior quality DNA from *Commiphora wightii* is tedious task due to existence of various secondary metabolites, polysaccharides and phenolic compounds. During present study the genomic DNA was isolated from dried leaf samples of four *C. wightii* genotypes. This procedure is applicable to fresh and old leaves of *C. wightii*. This protocol includes the application of 3% of CTAB and 1.5% PVP in the DNA extraction buffer. The use of CTAB in the DNA extraction buffer as it facilitates to disrupt the cell membrane (Bressan *et al.*, 2014). Polyvinylpyrrolidone (PVP) is an essential agent to eliminate the polyphenols by forming intricate hydrogen bonding with polyphenols and proficiently detach it from DNA (Kit and Chandran 2010). Autoclaved sand was used during grinding process to convert leaves into fine powder. Without sand

it was difficult to crush the leaves properly. The extraction and purification of DNA was performed in a single protocol. Good quality DNA obtained using the protocol (Table 1, Fig. 1). Obtained DNA was quantified using nanodrop-spectrophotometer. DNA concentration was ranged from 27.48 to 40.83 µg/µl and optical density (OD) was between 1.70 and 1.81. This method solved the problems of DNA degradation, contamination, and low yield due to binding and co-precipitation with starch and polysaccharides. The isolated DNA proved amenable to PCR amplification (Fig. 2). The technique is fast, reproducible, and can be applied for amplification of RAPD, ISSR and other molecular markers. The optimized DNA extraction buffer composed of higher quantity of CTAB as it makes complex with polysaccharides and fructans (Gawel and

Jarret 1991). Whereas other chemical PVP forms insoluble compounds with lactones and phenolics (Kim *et al.*, 1997). All these compounds are detached by precipitation for the duration of centrifugation after mixing with chloroform: isoamyl alcohol. Raising the quantity of β-mercaptoethanol from 0.2 to 0.3% helped to ensure the oxidation of phenolic compounds. Insertion of phenol in the protocol was avoided during application of chloroform: isoamyl alcohol solution to obtain high molecular weight DNA, as phenol might break phosphodiester bonds in DNA causing its degradation. A separate RNase A application, as recommended in numerous published protocols was incorporated in our extraction protocol and additional two times application of chloroform: isoamyl-alcohol (24:1) combination was competent to eliminate all impurities.

Table.1 Concentration and Optical Density of DNA isolated from dried leaves of *C. wightii*

S.	Label	Place of collection	Age of plant (years)	Concentration (µg/µl)	OD
1.	CW1	Morena	12	32.59	1.70
2.	CW2	Morena	12	40.83	1.74
3.	CW3	CAZRI, Jodhpur	8	31.58	1.73
4.	CW4	AAU, Anand	9	27.48	1.81

Fig.1

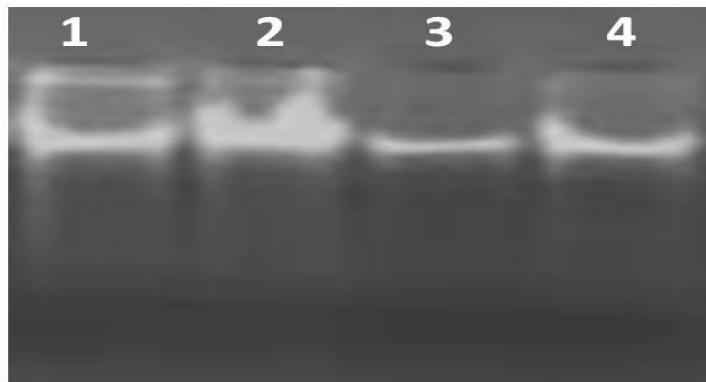


Fig. 1 DNA from dried leaf samples of *Commiphora wightii*

Fig.2

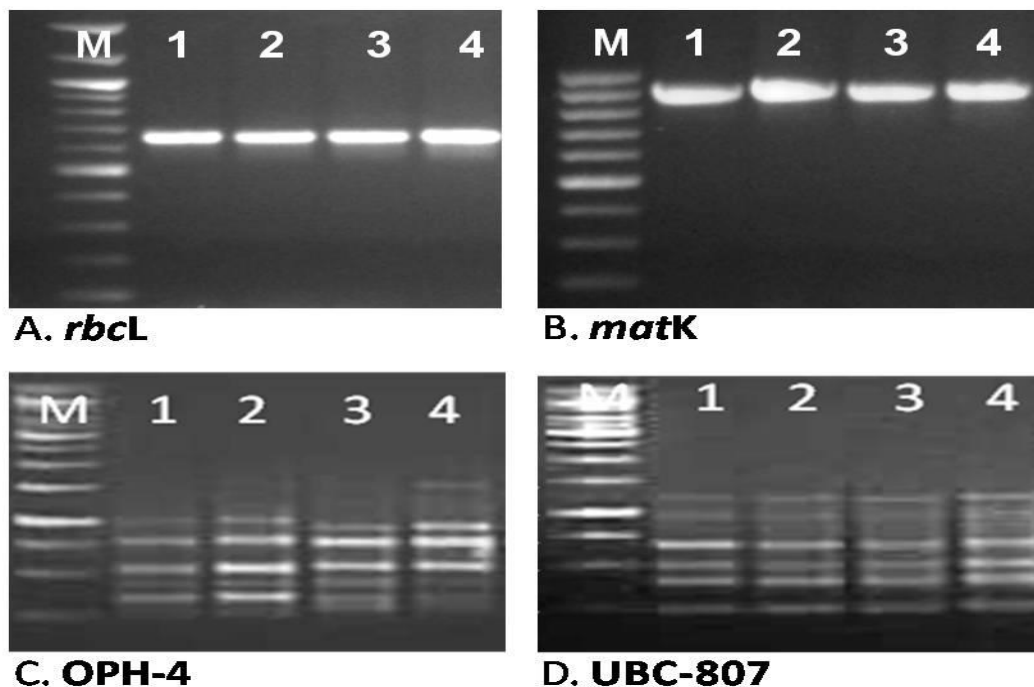


Fig. 2 Amplification pattern of *Commiphora wightii* DNA isolated from dried leaves

Application of sodium acetate removes the majority of secondary metabolites and polysaccharides from the DNA consequential in improved yield of high molecular weight of DNA. The DNA extracted by this protocol was used in the polymerase chain reaction with *rbcL*, *matK*, and ISSR and RAPD primers (Fig. 2). It was found that the PCR amplicons from successful reactions showed good quality bands with all primers. It indicates that the DNA extracted using this method was free from different secondary metabolites e.g. flavonoids, terpenes, and phenolic compounds, which hinder with the quality and yield of the DNA (Porebski *et al.*, 1997). It means secondary metabolites were effectively removed for the duration of the extraction procedure. Therefore, it confirms that the extracted DNA was appropriate for any analysis make use of PCR as a technique. The extracted DNA demonstrated acquiescent to PCR amplification. The developed protocol

is rapid and reproducible, producing good quality DNA for amplification of molecular markers.

Furthermore, we observed that the method detailed in this paper is efficient for plants that were recalcitrant to isolate the DNA. Finally, we trust that this protocol will be cooperative for DNA based molecular studies of various wild plant species with high level of secondary metabolites. On the basis of the results, it can be concluded that the present protocol gives genomic DNA with good quality and intactness; a spectrophotometric A_{260}/A_{280} value >1.81 . Furthermore, the protocol can be applied to extract DNA from young plant leaves as well as dried or frozen tissues. It is appropriate in situations when liquid nitrogen does not exist. The present protocol may also be used to other medicinal plants rich in polyphenolic compounds and polysaccharides.

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How to cite this article:

Sajjan Kumar Pooniya, Keerti Tantwai, Niraj Triphati and Sharad Tiwari. 2019. A Simple and Efficient Genomic DNA Extraction Protocol for Dried Leaf of Threatened Species *Commiphora wightii* (Arnott) Bhandari for Genetic Analysis of Plant Biological System. *Int.J.Curr.Microbiol.App.Sci*. 8(03): 1619-1624. doi: <https://doi.org/10.20546/ijcmas.2019.803.188>