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Extraction of high quality genomic DNA from *Madhuca longifolia* (J. Koenig ex L.) J.F. macbr.

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Abstract: *Madhuca longifolia* commonly known as the *Mahua* tree is a medium to large sized deciduous tree distributed in India, Nepal and Sri Lanka. It is found growing wild and cultivated. The tree is considered as a kalpavriksha by the tribal who are forest dwellers and keenly conserve this tree. The flowers and fruits are edible and the flowers are fermented to prepare *mahua* drink which is an integral part of their cultural heritage. The seeds are good source of edible fats and used to extract oil. Several pharmacological activities are reported using various parts of the tree. Very little information exists on the molecular aspects of *Madhuca longifolia* which requires high quality DNA. Optimization of DNA extraction using CTAB and SDS buffer system was standardized. A protocol for extraction of high quality DNA from tree species which are rich in polyphenols is hereby discussed.

Key words: *Madhuca longifolia;* Genomic DNA; CTAB; SDS; tree species.

Introduction

DNA fingerprinting studies has been significant in the fields of population genetics, genotypic diversity both inter-specific and intraspecific, plant systematics, construction of dendrogram for phylogenetic relationship and germplasm conservation [1]. DNA Extraction is the core element of any molecular biology experiment and is a pre-requisite step in the molecular marker based studies in plants. The quality, integrity, purity and quantity of extracted DNA influence the success of subsequent experiments. High throughput genotyping technologies and projects requiring screening of large number of samples require the use of fluorescence-labeled primers for PCR amplification. The use of such primers increases the sensitivity of the procedures but also requires a better quality of starting materials, including genomic DNA [2]. Most manufacturers recommend the use of highly purified genomic DNA requiring either the use of an expensive purification kit, a lengthy and complicated procedure or a faster method that reliably yield high-quality DNA. There is demand for rapid, simplified and inexpensive DNA extraction and/ or purification methods which can provide large amount of high quality DNA [3]. Several successful DNA extraction protocols have been developed but none of these are applicable to entire flora due to heterogeneity in the chemoprofile and genomic complexity of the plants [3]. The major problems faced during extraction of genomic DNA from plants are action of endonucleases, plant secondary metabolites such as polysaccharides and polyphenols and RNA contamination. Purified genomic DNA, often required for many applications in molecular studies is much more difficult to obtain from trees than other plants [4].

In higher plants especially in tree species secondary metabolites affects the extraction protocols as it tends to get accumulated with maturity of the plant parts. The major hurdles encountered by researchers in isolation and quantitation of high molecular weight genomic DNA from tree species include

- DNA degradation due to action of Endonucleases.
- Co- precipitation of inhibitory compounds such as polysaccharides and polyphenols.
- Low yield, RNA contaminated trailing, Poor A^0 260/280 ratio and improper primer binding due inferior quality DNA isolation.

Studies have shown that yield and quality of DNA often varied among species within same genera as well as among tissue types from the same trees [5]. Also there exists intraspecific chemical polymorphism in many varieties of plants, thus there is a necessity to customize DNA isolation protocols according to each plant and sometimes even plant tissues [6]. Since foliage and other tissues of trees often contain varying levels of tannins, polyphenols and polysaccharides, these impurities co-extract with DNA posing serious problems while obtaining genomic DNA. Polysaccharides co-precipitate with extracted DNA, forming highly viscous solution and also sometimes browning the pellet they also inhibit digestion with restriction digestion and *Taq* DNA polymerase activity, thereby hindering its application in DNA fingerprinting studies [7].

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Several methods are available and are being developed for isolating genomic DNA from plants [8, 9, 10, 11, 12]. However, a single isolation method is unlikely to be successful for different plants [13].

Materials and Methods

Plant Material

Sample of fresh young green leaves of *Madhuca longifolia var. latifolia* tree growing wild *in* Dahanu region, Dist. Palghar Maharashtra, and leaves of cultivated *Madhuca longifolia var. longifolia* were harvested for DNA extraction. These leaves were thoroughly washed with sterilized distilled water followed by 80% alcohol. Samples of each variety were stored at 4°C and were processed for DNA extraction.

Solutions:

Extraction buffer system 1: CTAB- 1% (w/v); Tris HCl (pH 8)- 100mM; NaCl – 1.4 M; EDTA (pH8)- 100mM.

Extraction buffer system 2: SDS 5% (w/v), Tris HCl (pH 8)- 100mM; NaCl – 1.4 M; EDTA (pH8)- 100mM; 3M Potassium acetate.

-Mercaptoethanol-4% (v/v); PVP- 5% (w/v), Phenol: Chloroform: Isoamyl alcohol (25:24:1) (v/v/v), Chloroform: Isoamylalcohol-24:1(v/v); TE buffer (10mM Tris HCl, pH8. 1mM EDTA); 70% and 80% Ethanol; Ribonuclease A (10mg/ml), Liquid nitrogen, Absolute alcohol (99.9%).

Homogenization of tissue/ plant part for DNA extraction

Plant tissues are very robust hence requires homogenization prior to DNA extraction. Several methods are employed for homogenization such as homogenizers or bead mills using glass or steel beads. In current research work grinding in chilled mortar and pestle was used in presence of liquid nitrogen. An attempt was also carried out to homogenize tissue in the absence of liquid nitrogen using absolute alcohol as a fixative [14, 15].

DNA Isolation

Fresh and dried leaves were surface sterilized with sterile distilled water followed by 80% ethanol. The leaves were cut into small pieces of size approx. 1 mm with sterile blade. The pre-chilled mortar and pestle was used to ground fresh (1g) and dried (1g) leaf samples. The homogenized leaf powder by the above two homogenized methods was scraped in to a dry microfuge tube and mixed with preheated buffer 5 ml CTAB and 5 ml of SDS buffers (added 10 µl Beta mercaptoethanol) and was incubated at 60°C for 1h. After incubation the mixture was cooled at room temperature and then equal volume of mixture of Equal volumes of Phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by chloroform: isoamyl alcohol (24:1) was added for removal of impurities.

Purification of DNA

The samples isolated using the above methods were purified as detailed. To each tube, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added, mixed for 3 minutes and centrifuged at 10000 rpm for 10 minutes at 4°C. to the aqueous phase chloroform: isoamyl alcohol (24:1) was added and the contents mixed by shaking for 3 minutes, followed by centrifugation at 10000 rpm for 10 minutes. The aqueous phase was transferred to a new tube and the above step was repeated thrice. 200 ml 1.4 M NaCl- TE was added to the old tube and shaken for 15 min. The old tube was centrifuged for 15 min at 12000 rpm. The aqueous phase was transferred to the new tube and mixed, followed by centrifugation at 12000 rpm for 15 min in order to settle any remaining debris. The supernatant was then transferred to a new tube.

Precipitation of DNA as pellet

Ice cold isopropanol (700 ml) was added to the sample and mixed gently and centrifuged at 10,000 rpm for 5 min and the supernatant discarded. The pellet was suspended in 600 µl of TE buffer, 60 µl 3M potassium acetate (pH 5.2) was added and DNA was precipitated by adding ice cold absolute isopropanol and incubated on ice for 20 min. (only for extraction buffer 2). Cold 70% ethanol and 5M NaCl was added to the pellet to wash it thrice and contents centrifuged at 5000 rpm for 5 min. The ethanol was discarded and the pellet air dried. The pellet was re-suspended in 200 ml 1X TE buffer and incubated overnight at 55°C.

RNase Treatment

The DNA was treated with DNase free Ribonuclease A. Large amounts of RNA in the sample can chelate Mg 2+ and reduce the yield of the PCR. This step removes RNA from the isolated genomic DNA. RNase (10 µl of 10 mg/ml) was added to 100 µl of re- suspended DNA pellet and then incubated at 37°C overnight. Equal volume of ice-cold absolute ethanol was added to each sample and then centrifuged at 10,000 rpm for 10 min to re-precipitate the DNA. This was done twice. The supernatant was poured off and the DNA pellets air-dried and finally the DNA pellet was dissolved in 50 µl of 1xTE buffer

Quantification of DNA

1 µl of DNA sample was diluted with 49 µl of 1X TE buffer (10 X dilution) and the optical density was measured at 260 nm against a 1X TE buffer in a Jasco Spectrophotometer. DNA concentration was then calculated according to the known method. Optical density (O.D) values were also taken at 280 nm (corresponding to protein), 230 nm (corresponding to RNA) and 320nm

(contamination). Total DNA purity was tested by a ratio of O.D values at 230: 260: 280.

Visualization of DNA

The DNA was examined for intactness using the gel electrophoresis method. Two microliter of the isolated DNA, 7 µl of sterile distilled water were mixed with 1.0 µl of 10 X loading dye and was loaded in a lane of 1.5% (w/v) agarose gel containing ethidium bromide for checking the quality of the DNA.

Results and Discussion

Table 1: DNA extraction using CTAB buffer and liquid nitrogen homogenization - Ratio and yield of two varieties of *Madhuca longifolia*

Sample	Ratio (260/280)	Ratio (260/230)	Yield (µg/gm)
Leaf A	1.831	0.858	652
Leaf B	1.856	0.692	534

Key:- *A- Madhuca longifolia var. latifolia*
B- Madhuca longifolia var. longifolia

Table 2: DNA extraction using CTAB buffer using absolute alcohol for homogenization - Ratio and yield of two varieties of *Madhuca longifolia*

Sample	Ratio (260/280)	Ratio (260/230)	Yield (µg/gm)
Leaf A	1.691	0.758	452
Leaf B	1.506	0.796	434

Key:- *A- Madhuca longifolia var. latifolia*
B- Madhuca longifolia var. longifolia

Table 3: DNA extraction using SDS buffer + Potassium acetate using liquid nitrogen for homogenization - Ratio and yield of two varieties of *Madhuca longifolia*

Sample	Ratio (260/280)	Ratio (260/230)	Yield (µg/gm)
Leaf A	1.731	0.738	512
Leaf B	1.801	0.696	514

Key:- *A- Madhuca longifolia var. latifolia*
B- Madhuca longifolia var. longifolia

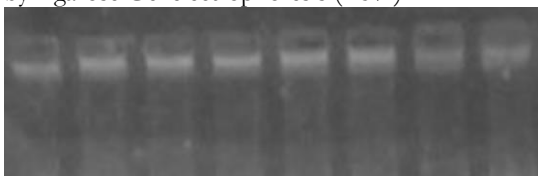
Table 4: DNA extraction using SDS buffer + Potassium acetate using absolute alcohol for homogenization - Ratio and yield of two varieties of *Madhuca longifolia*

Sample	Ratio (260/280)	Ratio (260/230)	Yield (µg/gm)
Leaf A	1.315	0.741	432
Leaf B	1.524	0.693	415

Key:- *A- Madhuca longifolia var. latifolia*
B- Madhuca longifolia var. longifolia

Polysaccharides and polyphenols generally cause degradation of DNA due to co-isolation of these interfering agents. With maturity of the tissue the amount of these agents elevates [7]. *Madhuca longifolia* varieties are rich in polyphenols and polysaccharides hence young green leaves were selected for DNA extraction. Homogenization of the leaf tissue is the crucial step in DNA extraction. Grinding in Liquid nitrogen and fixing of leaf tissue in absolute alcohol prior to grinding were the methods used for homogenization of leaf tissue. There was no significant difference in the yield and purity of DNA obtained the results showed that the ratio of A260/A280 was around 1.8 for CTAB and SDS buffer system with homogenization in liquid nitrogen and in a range of 1.3-1.6 when grinded in absolute alcohol. Similar results were reported for different plant species wherein good quality and better yield of genomic DNA can be obtained without the use of liquid nitrogen where its availability is limited. [14,15]. Concentration of CTAB in the extraction buffer was modified to 1% and that of SDS to 5%. Concentration of NaCl was modified to 1.4 M. Use of chemicals like beta mercaptoethanol and PVP enhanced the quality and yield of DNA. The chloroform: isoamyl alcohol step was repeated thrice with an additional washing with 70% ethyl alcohol after centrifugation to reduce protein contamination.

Figure 1: Genomic DNA extraction visualization by Agarose Gel electrophoresis (1.5%)



Legend: -
Lane 1 & 2 Liquid Nitrogen (Homogenization) + CTAB- Sample A & B
Lane 3 & 4 Liquid Nitrogen (Homogenization) + SDS+ K-acetate- Sample A & B
Lane 5 & 6 Alcohol (fixation) + CTAB- Sample A & B
Lane 7 & 8 Alcohol (fixation) + SDS- Sample A & B

Conclusion

Our results demonstrated that the optimized and modified protocol for DNA extraction using CTAB and SDS buffer gave high yields of genomic DNA from *Madhuca longifolia* species which was free from protein and RNA contamination. The addition of potassium acetate along with SDS buffer precipitated proteins giving a better extraction. Strong solution of NaCl, direct addition of insoluble PVP to the homogenized plant tissue resulted in better extraction. Use of absolute alcohol instead of liquid nitrogen for homogenization yielded similar results though not better than liquid nitrogen, alcohol can be used in absence of liquid nitrogen for homogenization. Phenol was used in the extraction procedure to remove interfering compounds which improved the overall yield. CTAB and SDS buffer yielded similar results however the modified CTAB protocol with grinding in liquid nitrogen was used for genomic DNA extraction. The extracted DNA was quantified and the purity and yield of extracted DNA was determined.

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