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OPTIMIZATION OF DNA EXTRACTION PROTOCOL FOR RAPD AND ISSR ANALYSIS IN *TECOMELLA UNDULATA*

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Genetic analysis of plants relies on high yield of pure DNA. Since, the leaf of *Tecomella undulata* accumulates large amounts of polysaccharides, polyphenols and secondary metabolites, which co-purify with genomic DNA thus leads to difficulty in extraction procedure. In the present study, experiments were carried out to extract high-quality amplifiable DNA by testing four DNA extraction methods for leaf tissues of *Tecomella undulata*. The key steps in the optimized protocol include increased incubation temperature, use of PVP and additives in the extraction buffer, repetition of purification step with CI and washing of DNA pellet with ethanol. The yield of DNA extracted was 209-560 µg/100mg of leaf tissue and the purity, evaluated by the ratio A_{260}/A_{280}, was 1.8-1.9, indicative of minimal levels of contaminating metabolites. The quality of the DNA extracted was confirmed by random amplification polymorphism DNA and by inter-simple sequence repeat amplification, proving that the DNA can be amplified via PCR.

Key words: DNA extraction, Inter-simple sequence repeat amplification Random amplification polymorphism DNA

Introduction

Tecomella undulata, member of Bignoniaceae family, is one of the important timber species of Rajasthan[1]. It has also occupied a reputed position of having valuable medicinal properties along with the ecological importance[2]. DNA extraction is a powerful and critical step for molecular analysis of any plant species. This process becomes more difficult if the species characterized with the high amount of secondary metabolites. PCR based molecular studies need high quality and pure genomic DNA, emphasizing screening of inexpensive, rapid and simple DNA extraction methods[3]. *Tecomella undulata* accumulates large amounts of polysaccharides, polyphenols and secondary metabolites which interfere greatly by reducing the yield and purity of DNA, thus causing no PCR products. The problems encountered include degradation of DNA due to endonucleases and co-isolation of highly viscous polysaccharides[4].

Since polyphenols and polysaccharides render genomic DNA unsuitable for molecular markers studies consequently it hinders PCR amplification by inhibiting the activity of *Taq DNA polymerase*[5-6]. An array of DNA isolation protocols[7-8] have been optimized and were used in combinations to extract quality DNA from plants. However, DNA extraction methods have not been compared for molecular marker analysis of *Tecomella undulata* whichis an economically important timber yielding tree species of arid and semi arid region. We report comparison of DNA extraction methods from leaf samples of *Tecomella undulata*and assess their suitability for analysis of Random Amplified Polymorphism DNA and Inter Simple Sequence Repeat (ISSR) marker system, which are useful for assessment of genetic diversity, phylogenetic relationship, gene tagging and high-density genome mapping [9].

Material and Methods

Genomic DNA was extracted from fresh leaves of mature trees of *Tecomella undulata* using four DNA extraction methods. Initially the available protocol for DNA isolation reported by Sharma et al., (2003) [10], classical method of Doyle and Doyle, (1987)[7] and modified Doyle and Doyle, (1990)[11] were used but satisfactory results were not obtained. Hence, few minor modifications were made in the modified protocol of Doyle and Doyle, (1990)[11] and the protocol was optimized. The detailed of the optimized method (Protocol 4) for genomic DNA extraction from leaf tissue of *Tecomella undulata* is described below

Standardized Protocol

Two gram of fresh leaf samples were homogenized in liquid N_2 using chilled pestle and mortar. The powdered material was transferred to 2.0 ml vial in which 1 ml of extraction buffer and 20 µl of β-mercaptoethanol was added. Mixture was then vortex and incubated in water bath at 65°C for 1 hour using floaters. After incubation 1ml of chloroform: isoamyl alcohol solution was added and vortexed. The solution was centrifuge at 10,000 rpm for 10 minutes at 25°C. The supernatant was collected into another vial and again centrifuge at 10,000 rpm for 10 minutes. The above steps were repeated if contamination appeared or pellet formed. 4 µl of RNase was added to the collected supernatant and incubated in the tubes at 37°C for 30 minutes with intermittent shaking and vortexing for every 5 min. Then again centrifuged the tube at 10,000 rpm for 10 minutes and supernatant was collected in a fresh tube. The DNA was then precipitated by adding an equal amount of ice cold isopropanol to the supernatant. The tubes were inverted for 2 minutes and then incubated the emulsion for overnight at -20°C. The tubes were centrifuged again at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with 200 µl of 70% ethanol. Thereafter 500 µl of 70% ethanol was added to the pellet and the tube was incubated at room temperature for 60 minutes. Again centrifuged at 10,000 rpm at 4°C for 10 minutes and the supernatant was discarded. After this, pellets were dried and resuspended in 200µl of TE buffer. The extracted DNA was finally stored at –20°C.

The extracted genomic DNA from all the four methods were then tested for purity index (A_{260}/A_{280} absorbance ratio) on UV- VIS Spectrophotometer (De Novix D11+ Spectrophotometer, USA) and for size, purity, and integrity on 0.8 % agarose gel at 100 V for 45 min to 1 h. A 1.8 (A_{260}/

A_{280}) ratio of extracted DNA samples indicates their high purity with values <1.8 or > 1.8 denoting contamination of proteins or RNAs[12].

RAPD and ISSR Analysis

The extracted genomic DNA was then tested for PCR amplification with RAPD (Operon) and ISSR (University of British Columbia, Canada) conducted using a thermocycler (Agilent Technologies Sure Cycler 8800, USA). For RAPD and ISSR assays, PCR amplifications were performed with a total reaction volume of 25 µl containing template DNA, 10 x *Taq* Buffer, 2 mM dNTP mix, 3U of *Taq polymerase* and molecular grade water.

The amplifications for ISSR assay were performed with the initial cycle of denaturation at 94°C for 5 minutes, followed by 39 cycles of denaturation at 94ºC for 45 seconds, annealing at 50°C for 45 seconds and extension at 72ºC for 2 minutes. For RAPD assay, PCR amplification was carried out at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94ºC for 1 minutes, annealing at 35°C for 1 minutes and extension at 72ºC for 2 minutes.

The amplified products were then mixed with 5 µl of 1X loading dye (bromophenol blue) and electrophoresis was carried out for 45 min at 100 V in 1.5% agarose gel with gene ruler of 100 bp plus DNA ladder as size marker. After electrophoresis, the gel was visualized and photographed under Gel Documentation System (Alpha Imager EP, Alpha Innotech, USA).

Results and Discussion

DNA analysis is the essential component for understanding the species at molecular level. For this high quality amplifiable DNA is prerequisite. Extraction of genomic DNA in *Tecomella undulata* is complicated due to the presence of polysaccharides, polyphenols and other secondary metabolites which greatly interfere with DNA resulting into poor amplification. Hence, experiments were carried out to isolate high-quality amplifiable DNA by testing four protocols using leaf tissue of *Tecomella undulata.*

Out of the various DNA extraction protocols tested, our protocol (protocol 4) was found suitable for extraction of desired quantity and quality of genomic DNA from leaf tissues (Table1; Fig.1). Approximately, 209-560 µg/100mg of leaf tissue was obtained with the optimized protocol which is good enough to be carried out RAPD and ISSR reactions. The key step in the optimized protocol included increased incubation temperature (65°C for 1 hour), use of higher concentration of PVP (3%), addition of additives (L ascorbic acid), and repetition of purification step with CI and washing of DNA pellet with ethanol. The protocol 1 reported by Sharma et al, 2003 includes use of CTAB precipitation to remove contaminants from DNA samples and exclude use of liquid nitrogen resulting into the low level of purity. The protocol 2 of Doyle and Doyle is based on lyses and purification with CTAB that selectively precipitates DNA while maintaining the solubility of many polysaccharides[13]. The purity ratio was also found to 1.7-2.59 resulting with cell debris contamination. The principal modification in this method was addition of higher concentration of PVP (3%) into the extraction buffer and pinch of PVP during crushing. PVP removes the polyphenols by forming the complex with polyphenols through hydrogen bonding allowing them to be separated from the DNA and reducing levels of polyphenols in the product. Adding low concentration of PVP into the buffer did not remove the polysaccharides and

polyphenolic contaminations completely. As a result of which these compounds get precipitated with DNA pellets and make them unsuitable for molecular markers studies. During the breaking down of cell walls polyphenols attach to DNA leads to irreversible oxidation of DNA and inhibition of enzymes resulting into the poor PCR amplification[14]. To avoid these oxidative effect of polyphenols, various anti-oxidant agents, such as PVP (polyvinylpyrrolidone), BSA (bovine serum albumin), or β-mercaptoethanol were used in the extraction buffer[15]. Some of the reported protocols tested in this study include these reagents in concentrations in the range from 0.2 to 2% β-mercaptoethanol[7,10,11].

In the optimized protocol, the concentration of β-mercaptoethanol was increased to 2%. Furthermore best results were obtained by the successive repetition of Chloroform: Isoamylalcohol step. Additional washing step of the extracted DNA pellets with wash buffer was also found useful for the removal of contaminates and clearance of DNA pellets.

Nevertheless, optimized CTAB based protocol 4 yielded excellent quality (purity ratio=1.8-2.0) and quantity (209-560 µg/100 mg of leaf tissue) of genomic DNA in *Tecomella undulata*. This method assured the extraction of pure DNA based on both quantitative (A_{260}/A_{280} and A_{260}/A_{230} ratios) and qualitative (color and viscosity) parameters. The recommend values for the A_{260}/A_{280} ratio should range from 1.8 to 2.0, and the optimized protocol was the only method that results in absorbance values within this interval (mean of 1.9), thereby characterizing high-quality DNA samples.

Higher values of absorbance ratios are the evidence of contamination by phenols while lower values indicate the presence of proteins once proteins absorb light at a wavelength of 280 nm[15].

S. No	Protocol	Yield (ng/ul)	Purity (A_{260}/A_{280})	Colour of the pellet
1	Protocol 1	31.21-103.84	1.67-2.21	Light to dark brown
2	Protocol 2	107-204.6	1.53-2.59	Yellow
3	Protocol 3	145.3-325	1.67-2.1	White
4	Protocol 4	209-560.7	1.8-2.0	White

Table 1: Yield and purity index of extracted genomic DNA from the four tested procedures on leaf tissues of *Tecomella undulate*

The extracted DNA using our optimized protocol (4) was tested in PCR amplification with RAPD and ISSR profiling with RAPD 11 and ISSR 15 primers based on our earlier experience. The PCR products produced for both the marker systems produced clear banding patterns (Fig 2). Thus, the protocol proved advantageous because of its simplicity and affordable reagents, besides achieving intact high molecular weight quality genomic DNA. The extracted DNA proved amenable to PCR amplifications including RAPD and ISSR analysis. The DNA extraction protocol described here consistently produced high yields of pure genomic DNA, while allowing for high sample throughout.

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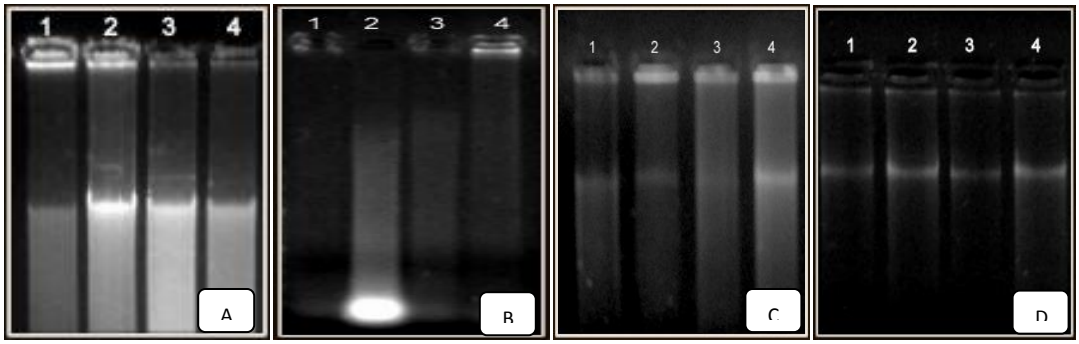

Fig 1: Gel images of different protocols tested for leaf tissue in *Tecomella undulata*
A) Sharma et al., 2003 B) Doyle and Doyle, 1987 C) Modified Doyle and Doyle, 1990
D) Optimized Protocol

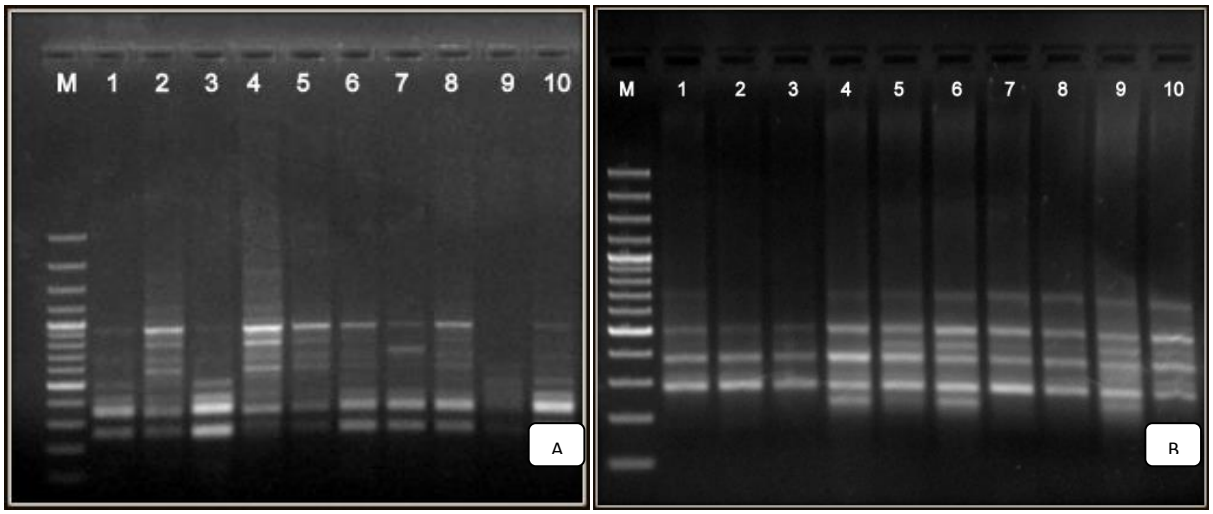

Fig.2 (A&B): A) RAPD pattern obtained from DNA extraction from optimized protocol. 10 DNA samples with primer RAPD-11 (lane 2-11). Lane1 Gene Ruler 100 bp Plus DNA ladder. B) ISSR pattern obtained from DNA extraction from optimized protocol. 10 DNA samples with primer ISSR-15 (lane 2-11). Lane 1 Gene Ruler 100 bp Plus DNA ladder

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