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GENOMIC DNA EXTRACTION FROM INNER BARK OF AN ECONOMICALLY IMPORTANT TREE SPECIES *SHOREA ROBUSTA* FOR RAPD STUDY

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ABSTRACT

For molecular-based studies, genomic DNA extraction from forestry tree species requires young leaf samples to obtain high-quality DNA. For some study, leaf samples must be collected from remote areas and are difficult to transport long distances in adverse conditions. In the present investigation, we have developed an alternative method of DNA extraction from the inner bark of *Shorea robusta*. For the removal of phenolic compounds, we used 2.5% polyvinylpyrrolidone (PVP) and added high sodium chloride concentrations (1.4M) to remove polysaccharides. The extracted DNA yield ranged from 7.9 to 16.7µg/100mg bark tissue and A_{260}/A_{280} ratio ranged from 1.7 to 1.87 and it gives positive amplification with PCR using RAPD (Random amplified polymorphic DNA) markers.

Keywords: Genomic DNA, RAPD, *Shorea robusta*, Inner Bark, Sodium chloride, Polyvinylpyrrolidone.

INTRODUCTION

Trees are important forest/ wild components and necessary for multiple habitats to preserve health. Sal (*Shorea robusta*) is a dipterocarpaceae tropical tree genus comprising three sub-families, 17 genera and 511 spp. (Ashton, 1982). *S. robusta* is an evergreen tree with a height of up to 50 m and a cylindrical bole that can be unbranched for up to 25 m with a diameter of up to 200 cm. It is native to the Indian subcontinent, where it serves many purposes, and can be found in East Asia. The younger tree has an elongated crown, but the crown becomes more rounded as the tree grows older. It is moderate to slow-growing, evergreen in wetter areas and deciduous in drier areas during the dry season. The bark is reddish brown and rough (Tewari, 1995). The distribution of *S. Robusta* was demarcated by Champion and Seth (1968). The forest cover ranging from Himachal Pradesh, Haryana, Uttar Pradesh, Bihar, West Bengal, Odisha, Madhya Pradesh, Chhattisgarh, Maharashtra, Jharkhand, Sikkim, Assam and Meghalaya extend from Uttarakhand in the north to Andhra Pradesh in the south and Tripura in the east. *S. robusta* is one of the most important timber-yielding trees in India, spread over an estimated area of 13 million hectares. Resin from *S. robusta* is known as Sal dammar or Indian dammar. It is used in Ayurvedic medicine as an astringent, burnt in Hindu ceremonies as incense, and used to caulk ships. *S. robusta* seeds and fruits are a source of vegetable fat and lamp oil. The seed oil is extracted from the seeds and used after processing as cooking oil (Panda, 2011). In view of all the above importance, conservation of this species becomes necessary. Now-a-days molecular biology play an important role in many aspects of species conservation.

Traditionally, molecular studies of plant species have used juvenile leaf tissues as the source of good quality of genomic DNA. The existing protocols suggested by Doyle and Doyle, 1990 and Stange *et al.,* (1998) are incapable for extracting DNA from adult leaf and wood tissues. Therefore, there was a need to optimize the protocols for DNA extraction from the inner bark tissue to yield high concentrations of good quality DNA fit for polymerase chain reaction (PCR) applications. In this article we reported a modified DNA extraction protocol that could produce relatively high quality DNA for molecular markers.

MATERIALS AND METHODS

Plant Material

Plant materials were collected from Sal forest near Doiwala, Dehradun (India). Total Genomic DNA was extracted from freshly collected inner bark of *S. Robusta.* (Table 1).

Requirements for DNA extraction

• CTAB extraction buffer (consisting of 0.1M Tris-HCl (pH-8.0), 0.2M EDTA (Ethylenediamine tetraacetic acid) (pH-8.0), 1.4M NaCl, 3% CTAB, 2.5% PVP (w/v) (added immediately before use), 0.005M Ascorbic acid (w/v) and 0.2% β-mercaptoethanol) (v/v).

• 5M Ammonium acetate solution (w/v) / Chloroform:

Isoamyl Alcohol (24:1) (v/v) / 76% and 96% Ethanol (v/v) / Isopropanol (-20ºC)/ TE Buffer consisting of (Tris HCl 0.01M (pH- 8.0), and EDTA 0.001M)/ Wash buffer (998 µl of 96% Ethanol and 2 µl of ammonium acetate solution)/ Liquid nitrogen/ Agarose powder/ EtBr and Bromophenol blue.

• Water bath/ Centrifuge/ Vortex/ UV transilluminator/ Agarose gel electrophoresis unit/ Bio photometer/ pH meter and weighing balance.

• 2 ml and 1.5 ml centrifuge tubes/ Micropipettes and tips.

• Conical flasks and measuring cylinder.

Requirements for Polymerase Chain Reaction

• PCR

• 0.2 ml PCR tubes/ Micropipettes and tips

Taq DNA polymerase/ $MgCl_2$/ Buffer/ dNTPs/ RAPD primer and template DNA.

Table 1. Geographical locations of the collected samples of S. Robusta.

Sample No.	Geographical details of ten individuals	
	Latitude (°N)	Longitude (°E)
1	30°12’56.50416”	78°7’27.04836”
2	30°12’56.10564”	78°7’27.17976”
3	30°12’56.322”	78°7’27.77412”
4	30°12’56.3832”	78°7’27.79248”
5	30°12’56.4966”	78°7’27.93036”
6	30°12’56.664”	78°7’27.65784”
7	30°12’56.85768”	78°7’27.282”
8	30°12’57.23028”	78°7’26.76072”
9	30°12’57.38292”	78°7’26.6304”
10	30°12’57.44592”	78°7’26.79708”

Standardized Extraction Methods

One ml of CTAB buffer with 2 µl β–mercaptoethanol was added in a 2 ml eppendorf tube and incubated in a water bath at 65°C for 15 minutes. The sliced and chopped bark tissue (100 mg) was ground to a fine powder with the help of a mortal pestle in the presence of liquid nitrogen and transferred to pre-incubated extraction buffer and incubated at 65°C for 1.5 hours (Kumar *et al.,* 2017). After incubation the samples were allowed to cool down to room temperature and then 500 µl of Chloroform: Isoamyl alcohol in the ratio of 24:1 was added to the sample and centrifuged at 6,000 rpm for 6 minutes at 4°C. The upper aqueous phase was pipetted out and transferred to a fresh micro centrifuge tube (1.5 ml). An equal volume of chilled isopropanol was added to the supernatant, mixed it well and incubated overnight at -20°C for better precipitation of genomic DNA. DNA was pelleted by centrifuging at 10,000 rpm for 10 minutes at 4°C. The pellet was washed with 998 µl of 96% alcohol and 2 µl of ammonium acetate. The pellet was again washed with 500 µl of 76% alcohol and centrifuged at 10,000 rpm for 6 minutes, after vacuum dried resuspended in 100 µl of TE buffer and stored at 4°C.

Qualitative and Quantitative analysis of extracted DNA

The yield of the extracted DNA was measured by UV-Visible spectrophotometer and purity was determined by calculating the absorbance ratio A_{260}/A_{280} nm (Table 2).The absorbance ratio of DNA sample within the range of 1.6-2 was considered as pure while above this range were considered contaminated with protein and those below by RNA (Aggarwal, 2008). The purity of DNA was checked by running the samples on 0.8% agarose gel (Figure 1).

Table 2. Quantitative analysis of genomic DNA extracted from inner bark of S. Robusta

Sample No.	DNA yield (µg/100 mg tissue)	A_{260}/A_{280} (ratio)
1	8.9	1.77
2	15.4	1.71
3	15.6	1.83
4	9.6	1.79
5	10.2	1.78
6	10.4	1.76
7	16.7	1.87
8	11.3	1.85
9	8.6	1.71
10	7.9	1.70

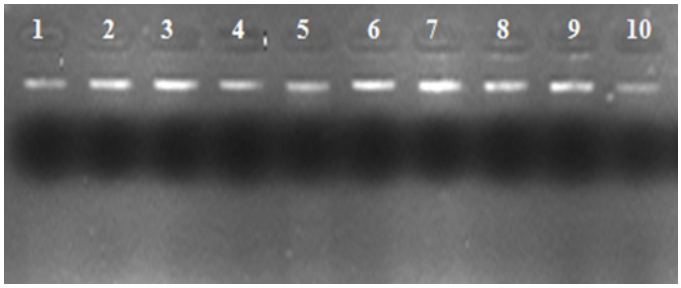

Figure 1. Genomic DNA extracted from inner bark of ten individuals (Lane 1-10) of *S. Robusta* on 0.8% agarose gel.

PCR amplification

RAPD primer (Mosseler *et al.,* 1992, M-182 Sequence: 5’GTT CTC GTGT3’ was used for the amplification. The reaction was carried out in a gradient PCR. Each 25 µl reaction mixture contained about 15 ng template DNA,

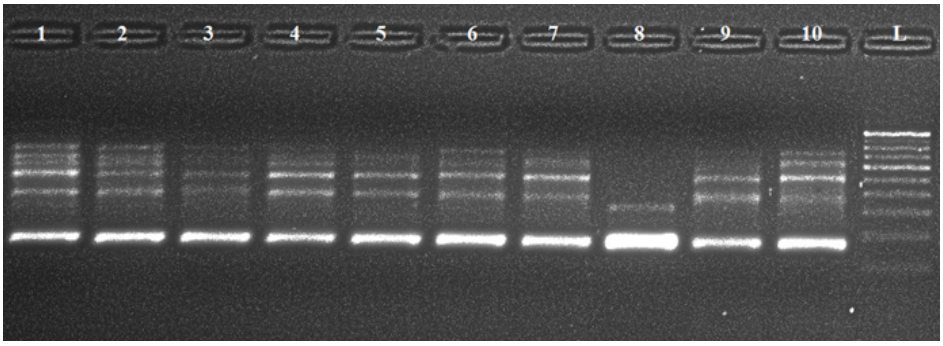

Figure 2. PCR amplification of extracted genomic DNA (1-10) with RAPD primer M-182 on 1.5% agarose gel. Bands were compared with the 100bp DNA ladder (Lane L)

1× PCR reaction buffer, 2 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.4µM primer and 1U of *Taq* DNA polymeras. The PCR amplification parameters consisted of one cycle at 94°C for 2 min; 41 cycles each at 94°C for 45 seconds; 37°C for 1 minute and 72°C for 1 minute followed by a final elongation at 72°C for 10 minutes. Amplification products were visualized on 1.5% agarose gel containing ethidium bromide (0.5 µg/ ml) run at 80 V for 1.5 hours (Figure. 2).

RESULTS AND DISCUSSION

Successfully high quality of DNA was extracted from the inner bark of ten individuals of *S. robusta*. The quality of extracted DNA was also high and A_{260}/A_{280} ratio (7.9 to 16.7), which is within the optimal sample range (Sambrook *et al.,* 1989). The fatty acid and proteins were the main components of the fresh wood of *S. robusta*. Fatty acid has lower density and non polar characteristic; it could easily be distinguished from the aqueous phase when it involved into centrifugation. The phenol and chloroform were frequently used for protein removing in custom DNA extraction method. It needed only a short centrifugation to separate DNA from all the other contaminants in our present protocol, for the most of proteins removed in the insoluble precipitate. The 1.4 M NaCl was added into extraction buffer to remove the polysaccharides by increasing their solubility in ethanol (Fung *et al*., 1992). In order to remove polyphenols from the inner bark, the PVP (2.5%) was added to the extraction buffer according to the result of Maliyakal (1992). The 5mM ascorbic acid and 0.2% β-mercaptoethanol were used to protect DNA against oxidation and degradation (Kumar *et al.,* 2015). The good amplification result of RAPD molecular markers also verified the good quality of these ten DNA samples. The outcomes indicate the usefulness of the protocol for the molecular marker investigations of the technique of DNA extracted from the inner bark of *S. Robusta.* It is extraordinarily beneficial for molecular studies regarding mature tall trees, where the collection of leaf tissue is hard.

CONCLUSION

All biological samples contain DNA. The major problems encountered in the isolation of pure DNA molecules are the degradation by mechanical damage or hydrolytic action of nucleases, contamination with RNA, proteins and polysaccharides. In this article we have described an alternative and modified DNA extraction method of plant tissue other than leaf that provides high quality DNA.

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