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OPTIMIZATION OF DNA EXTRACTION METHODS FOR SOME IMPORTANT FOREST TREE SPECIES

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ABSTRACT

The present investigation to selection of DNA extraction methods (1.Bousquet *et al*., 2.Cheng *et al.*, 3.Doyle and Doyle *et al.*, 4.Michiels *et al*., 5.Lin *et al*.,) for different types of plants, with suitable quantity and quality performance of DNA from several tree species (*Albizia procera* Benth., *Boswellia serrata* Roxb. var. serrata, *Dalbergia sissoo* Roxb., *Gmelina arborea* Roxb., *Jatropha curcas* Linn. and *Pongamia pinnata*). Quantity, quality tested by spectroscopy and amplification of extracted DNA by PCR with identical markers ISSR could vary among tree species according to the extraction method adopted.

Keywords: - DNA Extraction, Spectroscopy, Tree species, PCR, Molecular Marker, ISSR…etc**.**

1. INTRODUCTION

Extraction of DNA is still an important field of plant molecular biology. Molecular marker studies in plants, particularly for tree improvement, require large amounts of purified high molecular weight DNA. Projects that involve screening of large number of samples, such as evolutionary and breeding studies, require faster methods that reliably yield high-quality DNA (Csaikl *et al.*, 1988). Hence, there is demand for rapid, simplified and inexpensive DNA extraction/purification methods which can provide large amount of high quality DNA (Weising *et al.*, 1995). However, purified genomic DNA, often required for many applications in molecular genetic studies, is much more difficult to obtain from trees than other plants (Shepherd *et al.*, 2002). 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 (Henry *et al.*, 2001). 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. Such impurities also interfere in further DNA analysis. Polysaccharides inhibit restriction enzyme digestion and *Taq* DNA polymerase activity (Demeke and Adams, 1992; Fang *et al.*, 1992; Scott and Playford, 1996.). Polysaccharides often co-precipitate with extracted DNA, leading to formation of highly viscous solution (Do and Adams, 1991). The DNA is rendered unsuitable for restriction and Southern hybridization and often remains in the wells during electrophoresis. Consequently, many trees require highly complex extraction methods than do other annual plants (Scott and Playford, 1996).

Several methods are available and are being developed for isolating genomic DNA from plants. However, a single isolation method is unlikely to be successful for different plants (Loomis, 1974). Chemotypic heterogeneity among plants samples also would not allow optimal yield with a single protocol (Weising *et al.*, 1995), and hence, specific protocols need to be followed for different plants.

Albizia procera Benth., *Boswellia serrata* Roxb. var. *serrata*, *Dalbergia sissoo* Roxb., *Gmelina arborea* Roxb., *Jatropha curcas* Linn. and *Pongamia pinnata* Pierre are economically important tree species of industrial and medicinal value. These species have been particularly selected because of the recent interest in their large scale plantation and utilization. For example, *J. curcas* is presently a high priority species specially focused as a source of bio-fuel. *B. serrata* has been unsustainably exploited for its oleo-resin, googol. Hence, it is expected that studies at molecular level would be undertaken for assessing their diversity as well as breeding for improving their economically important traits. Such studies will need large quantities of high quality DNA. Comparative studies on evaluation of different DNA extraction methods for use in molecular marker studies in the above tree species have also not been reported. In the present study, five different DNA extraction and purification methods were evaluated for the above six economically important trees.

The relative yield and purity of genomic DNA extracted from six different plant tissues, which are potential sources of DNA, was also examined. The effect of DNA purification methods on endonuclease digestion with restriction enzyme and DNA amplification using ISSR (Inter Simple Sequence Repeat) primers was also examined.

2. Materials and Methods

2.1 Plants material

Fresh leaves obtained from different tree species viz., *A. procera*, *B. serrata*. var. *serrata*, *D. sissoo*, *G. arborea*, *J. curcas* and *P. pinnata* were used as sources of DNA (Plate 1). All the above tree species are located in the campus of Tropical Forest Research Institute, Jabalpur (Madhya Pradesh, India).

2.2 DNA extraction methods

Four methods based on CTAB and one method based on SDS for extraction of genomic DNA were compared. There were four replicates for each tree species for each method and experiments were repeated thrice.

2.2.1 DNA extraction method of Bousquet et al.

This method was originally described for extraction of DNA from tree species and was modified and adopted by Khasa et al. (1999) for isolation of microsatellites from alpine and western larch. The method was further modified in this study

2.2.2 DNA extraction method of Cheng et al.

This method was developed for tissues from woody species by Cheng et al. (1997). This method was found suitable for extraction of high quality DNA from different types of plant samples including bark, buds and leaves

2.2.3 DNA extraction method of Doyle and Doyle

This method described by Doyle and Doyle et al. (1987) is one of the most extensively followed methods for extraction of DNA from plants

2.2.4 DNA extraction method of Michiels et al.

This method was described by Michiels et al. (2003) as an optimized extraction protocol for extraction of DNA from latex containing plants belonging to family Cichorioideae.

2.2.5 DNA extraction method of Lin et al.

This method was described by Lin et al. (2001) for DNA minipreparation suitable for large-scale screening of transgenic plants.

2.3 DNA quantification and purity analysis

Genomic DNA from the leaf samples were quantified by measurement of sample absorbance at 260 nm with a spectrophotometer (UV-VIS Spectrophotometer 108; Systronics, India). The purity of genomic DNA was evaluated by measuring absorbance data ($A_{260/280}$ nm). The size, purity, and integrity of DNA were determined by horizontal agarose gel electrophoresis using system supplied by BioRad (Bombay, India), using 1% agarose in TBE buffer at constant voltage of 100V for 30 min to 1 hour. India).

2.4 Polymerase Chain Reaction and ISSR amplification

ISSR primer selection was based on studies, which are underway at Tropical Forest Research Institute, Jabalpur. ISSR primer UBC-834, obtained from University of British Columbia, Canada, was used in the study. The primer sequence was (AG) YT. PCR was done with a Palm Cycler (Corbett Research, Australia). DNA amplification was performed by adding 2μL of DNA to 8 μL reaction mix. Details of reaction mixture are given in Appendix. The PCR amplification consisted of: one cycle of 94˚C for 3 min; 30 cycle of 30 sec at 94˚C, 30 sec at 50˚C and 1 min at 72˚C; a final extension cycle of 10 min at 72˚C. Amplification products were visualized by horizontal agarose gel electrophoresis system (BioRad, Bombay) using 3%

agarose in TBE buffer at constant voltage of 100V for 2:30 hours.

2.5 Endonuclease digestion

Genomic DNA extracted from six tree species using five methods were incubated separately with 10 units of *Msp*I (Bangalore Genie, Bangalore, India) in the recommended buffer at 37^{o}C for 4 h. Restriction enzyme digestion was assayed by visual inspection after agarose electrophoresis using 2% agarose in TBE buffer at constant voltage of 100V for 1 h.

3. Results and discussion

DNA quantity

DNA quantity, extracted by five different methods, significantly varied among the six tree species investigated (Figs 1-6). Fresh leaves of *B. serrata* yielded maximum amount of DNA with overall mean of 1614.24 µg g^{-1} fresh leaf (Fig. 2) followed by *A. procera* with overall mean of 1357.24 µg g^{-1} fresh leaf (Fig.1). There was not much variation in quantities of DNA extracted from leaves of *D. sissoo, G. arborea, J. curcas* and *P. pinnata* (overall mean approx. 1000 µg g^{-1} fresh leaf) (Figs 3-6). Variation among samples of different tree species may be due to either intrinsic variation in terms of number and size of actively dividing cells or size of genome.

Extraction methods significantly influenced DNA quantity from the tree species investigated (Figs 1-6). The method of Lin et al. (2001) yielded maximum amount of DNA with overall mean of 1898.33 µg g^{-1} fresh leaf followed by the method of Cheng et al (1997) (overall mean = 1613.50 µg g^{-1} fresh leaf). DNA quantity was minimum in the method of Doyle and Doyle (1987) with overall mean of 444.44 µg g^{-1} fresh leaf (Figs 1-6).

Variation among extraction methods could be possibly due to varied composition of extraction buffers, varied components and parameters for precipitation and purification of DNA. For example, the method of Lin et al. (1997) uses SDS buffer for DNA extraction and comparatively few steps for completion of the entire extraction process. On the contrary, the method of Doyle and Doyle (1987) involves several time consuming extraction steps.

DNA quality

DNA quality (or purity) was examined by recording the absorbance of DNA preparations at 260 and 280 nm and computing A_{260}:A_{280} ratio. A_{260}:A_{280} ratio of more than 1.8 indicates high quality whereas values less than 1.8 indicate protein contamination. DNA extraction methods and tree species were significant sources of variation for quality of extracted DNA. Of the extraction methods tested, those of Bousquet et al, Michiels et al. and Cheng et al. yielded DNA of the highest quality with spectrophotometer absorbance ratio (A_{260}:A_{280}) of 1.76, 1.69 and 1.65 respectively (Figs 1-6). Doyle and Doyle method resulted in the lowest quality of DNA with absorbance ratio of 1.55. However, with this method, except for *B. serrata* and *D. sissoo*, which yielded DNA with high quantity of protein contamination, other trees yielded high quality DNA with absorbance ratio of

about 1.7. Among the tree species, *G. arborea*, *J. curcas* and *A. procera* consistently yielded DNA with high purity ratio (A_{260}:$A_{280} \geq 1.8$) with all the five methods tested. However, *B. serrata* yielded DNA with the lowest purity ratio (overall mean of 1.37) (Figs 1-6).

Variation in quality of DNA can be of two reasons: (1) Intrinsic ontogenetical, structural and biochemical variation among leaf samples of different tree species or (2) Variation in types of buffers used for extraction as well as the different steps of extraction with varying parameters and chemicals/reagents.

Endonuclease digestion

Restriction enzyme treatment of samples from all the tree species using all the five methods showed considerable amount of digestion (Figs 7-8).

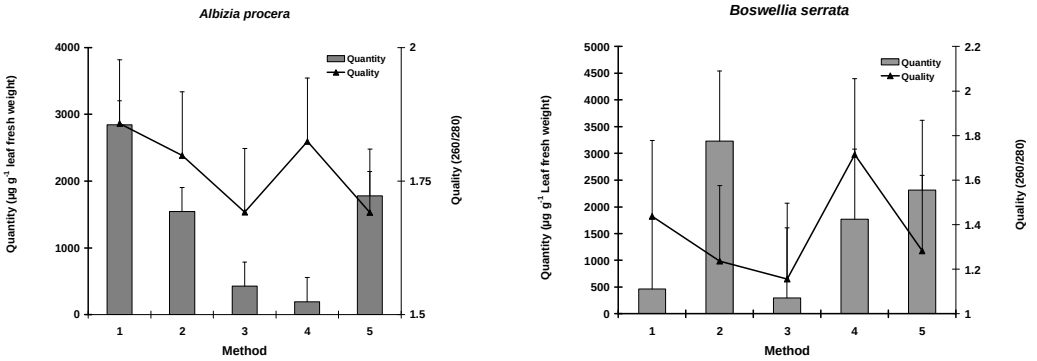
Time required for DNA extraction

Time and cost associated with DNA extraction and purification methods greatly influence marker associated studies, fingerprinting and genome mapping (Weising et al. 1995). The present study showed that there was variation in time required for different DNA extraction methods (please see Materials and Methods). The method of Lin et al. and Cheng et al. consisted of comparatively few steps for completion of the entire extraction process and were the most rapid extraction methods requiring less than three hours. On the contrary, the method of Bousquet et al., Cheng et al., Doyle and Doyle and Michiels et al. involved several time consuming extraction steps and took more than 16 hours to finish the entire processes.

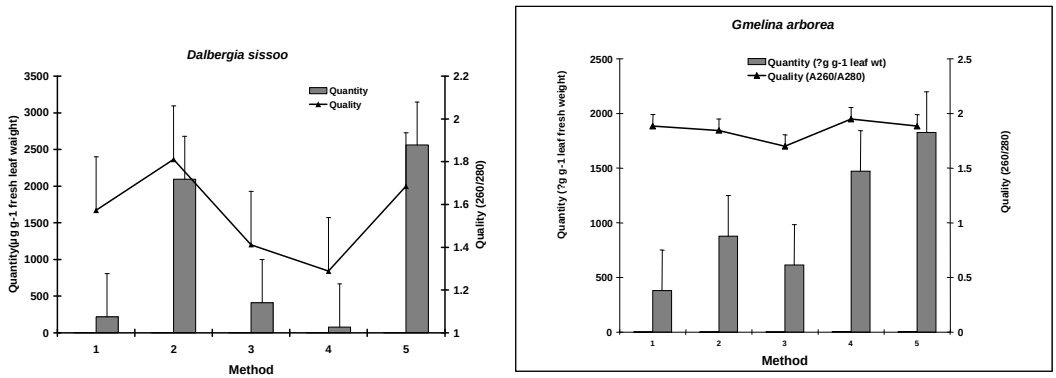
ISSR amplification

A major research focus in molecular marker studies is optimization of different parameters for PCR analysis (Weeden et al. 1992; Wolf et al. 1993; Staub et al. 1996). Quality and quantity of DNA are critical factors in molecular marker studies. Present study showed that DNA extraction methods and tree species influenced ISSR amplification of extracted DNA (Fig. 9). Among the five methods investigated, only the method of Bousquet et al. extracted amplifiable DNA from all the six tree species (Fig. 9). This method also produced DNA with high purity ratio 1.76 (Figs 1-6). The methods of Cheng et al., Doyle and Doyle and Michiels et al. could extract amplifiable DNA from *A. procera*, *D. sissoo*, *G. arborea*, *J. curcas* and *P. pinnata* (Fig. 9). Failure of DNA amplification from samples of *B. serrata* using these methods may be explained by the low purity ratio of these DNA samples indicating protein co-precipitation of extracted DNA. With reference to the method of Lin et al., DNA samples extracted through this method from *A. procera* and *G. arborea* only were amplified as shown in the ISSR assay (Fig. 9). This method extracted DNA with very low purity from *B. serrata* (1.28), *P. pinnata* (1.47) and *D. sissoo* (1.68) which could possibly the reason for absence of DNA amplification in these samples (Fig. 1-6). However, reason for failure of DNA amplification from samples of *J. curcas* which had high purity ratio (1.82), is not

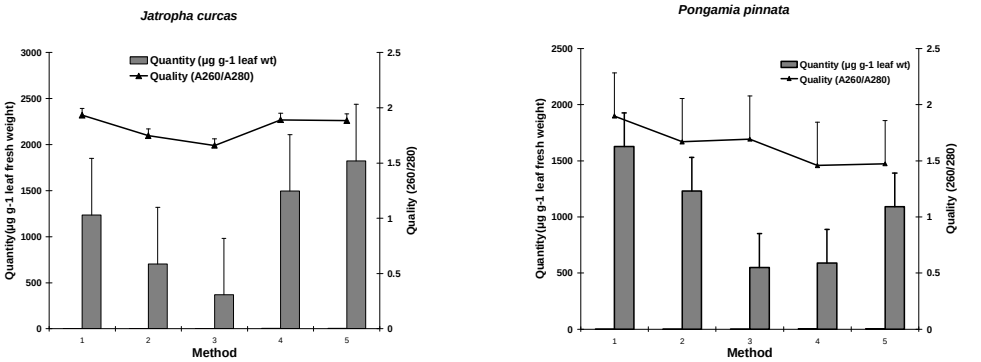
clearly understood. It is possible that such samples, even with high purity ratio, may still have trace levels of co-precipitation of phenols or other secondary metablolites, which could not be removed by the extraction method like that of Lin et al. It is important to note that all the other DNA samples from this species extracted by the other methods showed ISSR amplification.



1. Bousquet et al; 2. Cheng et al; 3.Doyle and Doyle et al; 4. Michiels et al; 5.Lin et al



1.Bousquet et al; 2. Cheng et al; 3.Doyle & Doyle et al; 4. Michiels et al; 5.Lin et al



1. Bousquet et al; 2. Cheng et al; 3.Doyle and Doyle et al; 4. Michiels et al; 5.Lin et al

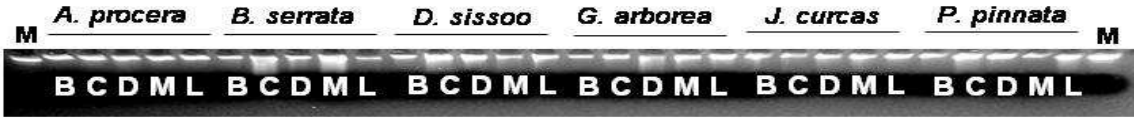


Fig. 7 Genomic DNA from different tree species extracted by different methods. Lanes are marked with letters corresponding to the methods. B - Bousquet et al.; C - Cheng et al.; D – Doyle and Doyle; M – Michiels; L – Lin et al. M – Marker;

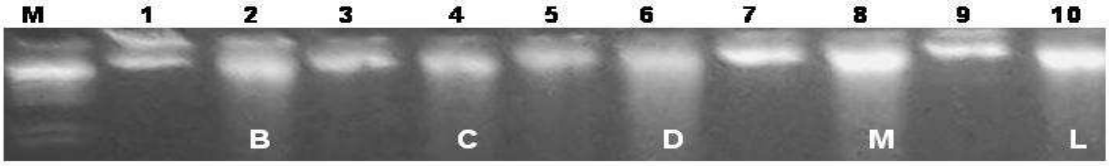


Fig. 8 Restriction enzyme treatment of genomic DNA of *Albizia procera* extracted by different methods. Lanes - 1,3,5,7,9 Uncut DNA; Lane 2, 4, 6, 8, 10 marked with letters corresponding to the methods. B - Bousquet et al.; C - Cheng et al.; D – Doyle and Doyle; M – Michiels; L – Lin et al. M - Marker;

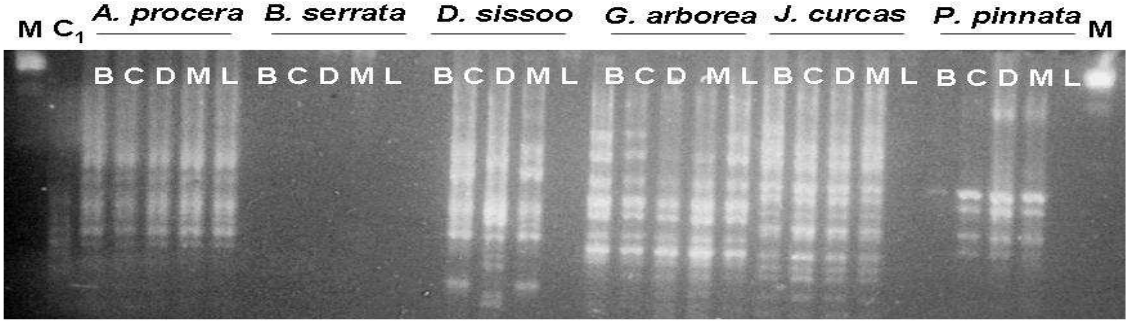


Fig. 9 ISSR amplification of genomic DNA from different tree species using primer UBC-834. M – Marker; C_1 - Control (without DNA); Lanes are marked with letters corresponding to the DNA extraction methods. B - Bousquet et al.; C - Cheng et al.; D – Doyle and Doyle; M – Michiels; L – Lin et al.

4. CONCLUSION

The present investigation clearly demonstrated the need for selection of appropriate DNA extraction methods for different tree species. A single extraction method may not be suitable for extraction of DNA with suitable quantity and quality from several tree species. Quantity, quality and amplification of extracted DNA could vary among tree species according to the extraction method adopted.

By taking into consideration the important factors viz., quantity, quality, suitability for ISSR amplification as well as the total time required for extraction, among the five extraction methods investigated, the modified method of Cheng et al. was found the best method for tree species viz., *A. procera, B. serrata, D. sissoo, G. arborea, J. curcas* and *P. pinnata*. This method extracted fairly high quantity of DNA (1613 mg g-1 fresh leaf) and took less than three hours to complete the entire procedure. The methods of Doyle and Doyle and Michiels et al. were also found

good to extract amplifiable DNA from *A. procera*, *B. serrata*, *D. sissoo*, *G. arborea*, *J. curcas* and *P. pinnata*, but these took long time of more than one day to complete the entire extraction processes.

For *B. serrata*, the method of Bousquet el al. emerged as the only procedure capable of producing amplifiable DNA. While the methods of Cheng et al., Michiels et al. and Lin et al. extracted high amounts of DNA from this species, the extracted DNA contained high amount of protein and other contaminants as indicated by the poor purity levels. In such case, the quality of DNA can be improved by including more detailed steps involving high levels of PVP and Proteinase K.

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