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Full Length Research Paper

Optimization of DNA extraction for ISSR studies in *Tectona grandis* L.f. - an important forest tree species.

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Four extraction methods and sample types were evaluated for yield, quality and suitability of genomic DNA for ISSR marker amplification in teak (*Tectona grandis*). Both CTAB and SDS based extraction procedures extracted better quantity and purity index of genomic DNA. Leaves of trees, bud grafts and seedlings yielded better DNA than seeds. Using identical PCR conditions, DNA extraction methods and sample types influenced amplifications of ISSR markers, with no amplifications among seed samples. DNA extraction method and sample type are very important consideration for reproducible ISSR-based molecular marker analysis in teak.

Key words: DNA extraction, ISSR, CTAB, SDS, teak.

INTRODUCTION

Molecular marker studies require large amount of quality genomic DNA, emphasizing screening of inexpensive, rapid and simple DNA extraction methods (Weishing et al., 1995). Yield and quality of DNA often varies among tree tissue types (Henry, 2001). Besides, purification of genomic DNA in trees is difficult due to co-extraction of high quantities of tannins, polyphenols and polysaccharides (Shepherd et al., 2002). Since polysaccharides render genomic DNA unsuitable for restriction/Southern hybridization and inhibit PCR amplification by Taq DNA polymerase, many trees require highly complex extraction methods (Scott and Playford, 1996). However, DNA extraction methods and sample types have not been compared for molecular marker analysis of teak (*Tectona grandis*, 2n=36, Family: Verbenaceae), which provides premium timber in India, Myanmar, Laos, Thailand and Indonesia (Troup, 1921) with large scale plantations in Asia, Africa and Central America (Kertadikara and Prat, 1995).

We report comparison of DNA extraction methods for different leaves and whole seeds of teak and assess their suitability for analysis of Inter Simple Sequence Repeat (ISSR) markers, which are useful for assessment of genetic diversity, phylogenetic relationship, gene tagging and high-density genome mapping (Godwin et al., 1997).

MATERIAL AND METHODS

Genomic DNA was extracted from fresh leaves (one month-old seedlings, 10-12 year-old trees and bud grafts from 40-50 year old plus trees) and seeds of teak using four extraction methods. Among them, Method #1 (Doyle and Doyle, 1990) and Method #2 (Porebski et al., 1997) were based on CTAB and Method #3 (Plaschke et al., 1995) and Method #4 (Lin et al., 2001) based on SDS. Porebski et al. (1997) also used high salt concentration and PVP, and Plaschke et al. (1995) sodium bisulfite in their methods. The extracted genomic DNA was tested for purity index (A_{260}/A_{280} absorbance ratio) on UV-VIS Spectrophotometer (Systronics, India) and for size, purity, and integrity on 1% agarose gel at 100 V for 30 min to 1 h. A 1.8 (A_{260}nm/ A_{280}nm) ratio of extracted DNA samples indicates their high purity with values <1.8 or > 1.8 denoting contamination of proteins or RNAs (Sambrook et al., 1989).

For ISSR marker analysis, Primer UBC-834 [$(AG)_8$ YT] from University of British Columbia, Canada was used on the basis of our previous experience. PCR for ISSR marker amplification was performed on a PalmCycler (Corbett Research, Australia), incorporating 2 µL (30 ng) genomic DNA to a 8 µL reaction mix containing 1X PCR buffer, 0.1 mM each of dNTPs, 2.5 mM $MgCl_2$,

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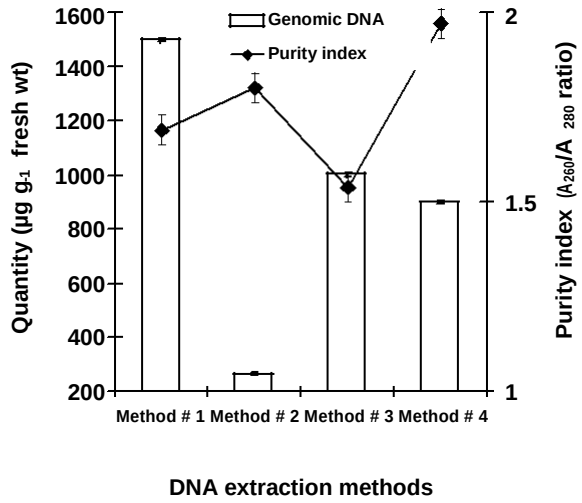

Figure 1. Quantity and purity index of genomic DNA of teak (*Tectona grandis* L. f.) as influenced by different methods (see Table 1). Data are mean of three replicates and vertical bars represent values of critical difference at p=0.01.

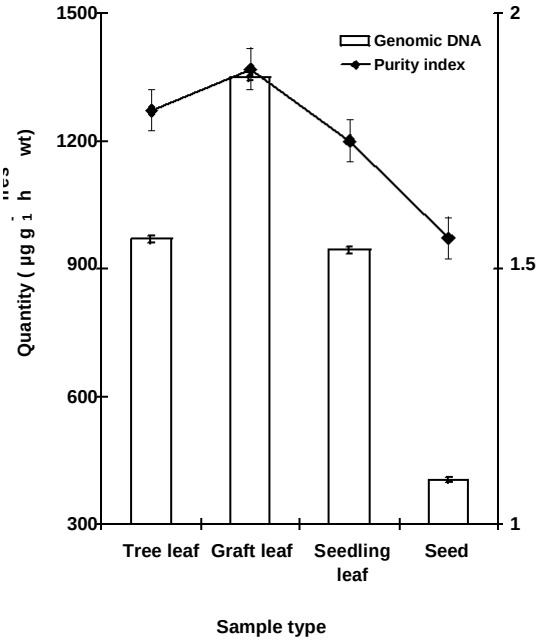

Figure 2. Quantity and purity index of genomic DNA of teak (*Tectona grandis* L. f.) in different sample types. Data are mean of three replicates and vertical bars represent values of critical difference at p=0.01.

0.8 µM primer and 1.0 unit of Taq polymerase. The PCR amplification programme consisted of: one cycle of 94^{o}C for 3 min; 30 cycles each of 30 sec at 94^{o}C, 30 s at 50^{o}C and 1 min at 72^{o}C; a final extension cycle of 10 min at 72^{o}C. Amplification products were visualized on 2% agarose gel at 100 V for 2.5 h.

RESULTS AND DISCUSSION

DNA extraction methods (Figure 1), type of samples (Figure 2) and their combinations (Table 1) significantly influenced yield and quality of genomic DNA. CTAB-Method #1 yielded the highest quantities of DNA, which was about 1.5 times more than those of SDS- Methods #3 and #4. However, CTAB-Method #2 including high salt ions and PVP drastically decreased genomic DNA yield by 5.63, 3.77 and 3.38 times in comparison to Methods #1, #3 and #4, respectively. Seemingly, lengthy and cumbersome steps involved in Method #2 for removal of polysaccharides, polyphenols and tannins as major impurities (Porebski et al., 1997) must have lost much of genomic DNA. Nevertheless, CTAB-Method #2 yielded genomic DNA of the highest purity index. On the other hand, SDS-Methods #3 and #4 provided protein-contaminated genomic DNA (Figure 1).

Further, fresh leaves (irrespective of sources) extracted better yield (2.33–3.33 times) and quality of genomic DNA than seeds. It may not be out of place to mention that DNA yields depend upon number of nuclei per unit area or weight of plant sample, which are always more in growing organs such as leaves, shoots, roots and meristems than resting structures like rhizomes, corms, bulbs and seeds. Besides, cells of seeds are loaded with reserved foods such as carbohydrates, lipids and proteins, accounting for both low yield and quality of genomic DNA. Interestingly, leaves from bud grafts yielded 1.39-1.43 times higher quantities of RNA-contaminated genomic DNA than leaves from other sources (Figure 2). This is plausible because a graft represents a union of two separate genotypes, which sets both competition and compatibility among themselves, resulting in fast growth (cell division) and high metabolic turnover requiring *de novo* synthesis of transcripts (RNA).

As for interactions between methods and type of samples (Table 1), Methods #1 and #4 extracted the highest genomic DNA from fresh leaves of bud grafts. The extracted DNA by Method #1 was also of adequately high purity index and that by Method #4 of heavily contaminated with RNA. In contrast, Method #2 invariably extracted low genomic DNA from all sample types. Further, irrespective of the methods used, the seed samples exhibited poor yield with large protein contaminations, registering minimum value with Method #4. Of these, Method #3 was initially devised for extraction of genomic DNA from wheat seeds with carbohydrates as predominantly reserved food (Plaschke et al., 1995). The same method resulted in poor yield of protein-contaminated genomic DNA from lipid and protein rich teak seeds.

Time and cost associated with DNA extraction and purification methods greatly influence molecular diversity analysis, fingerprinting and genome mapping (Weishing et al., 1995). In the present study, Method #1 requiring 3-4 h for extraction of genomic DNA emerged to be highly

Table 1. Quantity and purity index of genomic DNA of teak (*Tectona grandis*) obtained from four sample types using four different methods; Doyle and Doyle (Method #1), Porebski et al. (Method #2), Plaschke et al. (Method #3) and Lin et al. (Method #4).

Sample type	Method	Genomic DNA	
		Quantity (µg g^{-1} fresh wt)	Purity index (A_{260}/A_{280} ratio)
Tree leaf	Method # 1	1570	1.77
	Method # 2	190	1.78
	Method # 3	1343	1.87
	Method # 4	780	1.83
Graft leaf	Method # 1	1950	1.62
	Method # 2	240	1.89
	Method # 3	1271	1.67
	Method # 4	1930	2.40
Seedling leaf	Method # 1	1620	1.74
	Method # 2	420	1.85
	Method # 3	940	1.26
	Method # 4	790	2.14
Seed	Method # 1	850	1.64
	Method # 2	210	1.69
	Method # 3	454	1.37
	Method # 4	100	1.52
$CD_{0.01}$		13	0.06

$CD_{0.01}$= Critical Difference at $p = 0.01$.
Data are mean of three replicates.

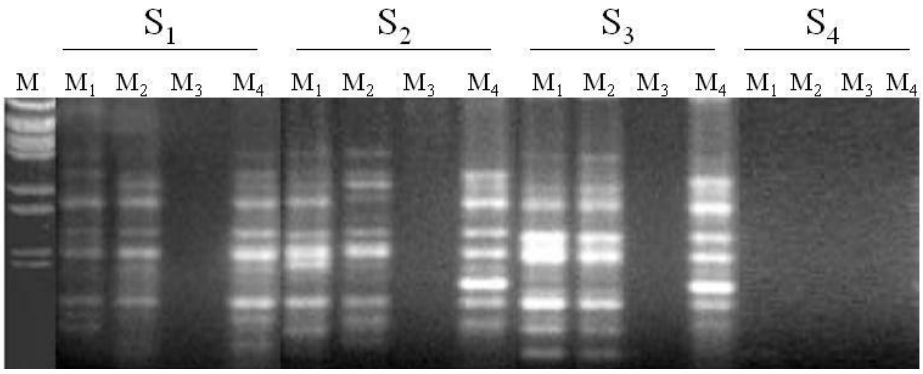

Figure 3. Ethidium bromide stained agarose gel showing PCR-ISSR products after amplification of genomic DNA extracted from four sample types (S_1 - tree leaf, S_2 - graft leaf, S_3 - seedling leaf, S_4 - seeds) using four Methods (M_1 – Method #1, M_2 - Method #2., M_3 - Method #3, M_4 - Method #4), M – DNA ladder.

promising compared to some of the quick procedures including Methods #3 and #4 requiring < 2 h. Method #2 was the most time consuming protocol, which took nearly two days to complete all extraction steps. Similarly, quantity and purity of extracted genomic DNA also plays crucial role for analysis of molecular diversity and optimization of different parameters for PCR (Weeden et al., 1992; Staub et al., 1996).

Sample types and extraction methods significantly influenced amplification pattern of ISSR markers (Figure 3). Among the four methods used, Methods #1, #2 and #4 exhibited PCR-ISSR amplification, but Method #3 failed to support PCR amplification of genomic DNA. High protein contamination in extracted DNA by Method #3 (Table 1) may be responsible for failure of PCR amplification. CTAB-Method #2 and SDS-Method #4 maximally produced an average of 7.3 and 7 ISSR amplicons, respectively. CTAB- Method #1 also yielded

an average of 5.3 ISSR amplicons. The higher purity index in Method #2 (A_{260}/A_{280} = 1.8) than Method #1 (A_{260}/A_{280} = 1.7) of extracted DNA possibly explains maximum number of amplicons in the former.

In addition, DNA extracted from seedling leaves yielded maximum 6 amplicons, followed by tree leaf (4.75 amplicons) and graft leaf (4 amplicons) (Figure 3). Lower number of amplicons in extracted DNA of tree/graft leaf suggests presence of contaminants like polysaccharide and polyphenols as well as RNA, which inhibits *Taq* polymerase (Scott and Playford, 1996). The failure of amplification of protein-contaminated DNA from seeds testifies this argument. Hence, DNA quality is a major factor in genetic analysis of teak using molecular markers like ISSR and confirms earlier reports on other plants (Weeden et al., 1992; Staub et al., 1996). Accordingly, the extraction methods must appropriately include steps to remove contaminations of proteins by Proteinase K and RNAs by RNase.

The above results have significant practical implications in molecular marker studies, for the low quality of DNA obtained from particular tissue-extraction method combinations affects analysis and interpretation of genetic differences between individuals in segregating populations. Hence, a judicious selection of DNA extraction methodology and sample source is required for reliable DNA based marker studies in teak. It is clear from the literature that different plant samples yield DNA of varied quantity and quality with different extraction and purification methods. Very often in case of forest trees, only one extraction method has been followed for most of the studies using different plant samples such as young/mature leaves, seeds, megagametophytes, calli, etc. Nevertheless, some studies comparing various extraction methods of DNA have been carried out in vegetable and other crops (Csaikl et al., 1998; Boiteux et al., 1999; Henry, 2001). Our investigation demonstrates that DNA extraction procedure and sample type in teak significantly affect yield and quality as well as ISSR amplification.

Considering extraction time, DNA yield and purity, DNA quantity and PCR-ISSR amplifications, Methods #2 (CTAB) and #4 (SDS) emerged to be the best choice for molecular diversity analysis of teak. However, Method #2 needs little modification for economy of time. Further, Method #4 extracting genomic DNA with RNA contamination (purity index 1.97) did not interfere with the ISSR amplification, but may be further improved using an additional step of RNase treatment of the DNA.

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