

Full Length Research Paper

Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India

Padmalatha K, Prasad M.N.V*

Department of Plant Sciences, University of Hyderabad, Hyderabad, Andhra Pradesh, 500 046, India.

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Genetic analysis of plants relies on high yields of pure DNA samples. Here we present the optimization of DNA isolation and PCR conditions for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India containing high levels of polysaccharides, polyphenols and secondary metabolites. The method involves a modified CTAB extraction employing polyvinyl pyrrolidone while grinding, successive long-term Chloroform : Isoamylalcohol extractions, an overnight RNase treatment with all steps carried out at room temperature. The yield of DNA ranged from 1-2 µg/µl per gram of the leaf tissue and the purity (ratio) was between 1.6-1.7 indicating minimal levels of contaminating metabolites. The technique is ideal for isolation of DNA from different plant species and the DNA isolated was used for randomly amplified polymorphic DNA (RAPD) analysis. RAPD protocol was optimized based on the use of higher concentration of MgCl₂ (3 mM), lower concentrations of primer (0.5 µM) and Taq polymerase (0.2 units), 50 ng of template DNA and an annealing temperature of 37°C, resulted optimal amplification. Reproducible amplifiable products were observed in all PCR reactions. Thus the results indicate that the optimized protocol for DNA isolation and PCR was amenable to plant species belonging to different genera which is suitable for further work on diversity analysis.

Key words: *Vitex pubescens*, *Nervilia aragoana*, *Gymnema sylvestre*, *Withania somnifera*, *Origanum majorana*, *Boswellia serrata*, *Saraca asoca*, *Gloriosa superba*, polysaccharides, PCR amplification.

INTRODUCTION

Medicinal and aromatic plants (MAPS) utilization and conservation has attracted global attention (Parrotta, 2001). Several of these MAPS contain exceptionally high amounts of polysaccharides, polyphenols, tannins,

hydrocolloids (sugars and carragenans) and other secondary metabolites such as alkaloids, flavanoids phenols, terpenes and quinines which would interfere with the DNA isolation procedures.

The problems encountered in the isolation and purification of DNA specially from MAPS include degradation of DNA due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Moreover, the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993), interference with DNA amplification involving random primers, e.g. RAPD analysis (Mejjad et al., 1994), and improper priming of DNA templates during

*Corresponding author. E-mail: mnvsl@uohyd.ernet.in
Fax: 91-40-23010145

Abbreviations: CTAB, Hexadecyltrimethylammonium bromide; Taq, *Thermus aquaticus*; TBE, Tris borate-EDTA; TE, Tris-EDTA; PCR, polymerase chain reaction; PVP, Polyvinylpyrrolidone; RAPD, Random amplified polymorphic DNA, RT, Room temperature, O/N, Overnight.

thermal cycle sequencing. Different plant taxa often may not permit optimal DNA yields from one isolation protocol. For example, some closely related species of the same genus require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required. Various protocols for DNA extraction have been successfully applied to many plant species (Doyle and Doyle, 1987; Ziegenhagen et al., 1993), which were further modified to provide DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). We have tested previously established DNA isolation protocols but these methods resulted in DNA with lot of impurities and not very suitable for RAPD analysis

Therefore, we report here a total genomic DNA isolation protocol derived from a method originally developed for other plants (Doyle and Doyle, 1987). Modifications were made to minimize polysaccharide co-isolation and to simplify the procedure for processing large number of samples. The protocol optimized for RAPD proved to be inexpensive with relation to the use of primer, quantity of DNA, usage of dNTPs, *Taq* polymerase and the reaction volume. Thus the protocol derived for both genomic DNA isolation and RAPDs, is genus independent, efficient, inexpensive, simple, rapid and yields pure DNA amplifiable by PCR as indicated by the results of the RAPD technique. The isolated DNA would be suitable for further downstream applications.

MATERIALS AND METHODS

Plant material

The eight plant species; 1. *Vitex pubescens* (Verbenaceae), 2. *Nervilia aragoana*. (Orchidaceae), 3. *Gymnema sylvestre* (Asclepiadeceae), 4. *Withania somnifera* (Solanaceae) 5. *Origanum majorana* (Lamiaceae), 6. *Boswellia serrata* (Burseraceae), 7. *Saraca asoca* (Caesalpinaceae) and 8. *Gloriosa superba* (Liliaceae) were collected from various forests in the state of Andhra Pradesh, India (2001-2003) and grown in experimental site. After acclimatization 1 g of young leaves were harvested fresh for DNA isolation.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), Tris HCl pH 8.0 (0.5 M); EDTA pH 8.0 (0.5 M); NaCl (5.0 M), PVP (0.1 g/1 g of leaf tissue, added while grinding) was prepared. 3 M Sodium acetate solution (pH 5.2), ribonuclease A (10 mg/ml), Chloroform : Isoamylalcohol (24:1), Phenol:Chloroform:Isoamylalcohol (25:24:1v/v/v), Ethanol (70%, 100%) and TE buffer (Tris HCl, 10 mM, EDTA, 1 mM, pH 8.0) are the additional solutions required.

DNA isolation protocol

1. Freshly harvested leaf sample (1 g) was ground in liquid nitrogen using a mortar and pestle along with 0.1 g of PVP. The pulverized leaves were quickly transferred to 3 ml of freshly prepared prewarmed (65°C) extraction buffer and shaken vigorously by in-

version to form slurry. The tubes were incubated at 65°C in hot air oven or water bath for 60-90 min with intermittent shaking and swirling for every 30 min.

2. An equal volume of Chloroform : Isoamylalcohol (24:1) was added and mixed properly by inversion for 30 min and centrifuged at 12,000 rpm for 15 min at RT (room temperature) to separate the phases (long term mixing of samples in Chloroform:Isoamylalcohol approximately for 30 min, will help in removal of pigments and formation of brownish colour in DNA sample can be omitted).

3. The supernatant was carefully decanted and transferred to a new tube and was precipitated with equal volumes of cold Isopropanol, and gently mixed to produce fibrous DNA and incubated at -20°C for a minimum of 30 min.

4. The samples were centrifuged at 12000 rpm for 15 min. The pellet was washed with 70% ethanol, air dried and resuspended in 3 ml of TE buffer and 5 µl of RNase was added and incubated O/N at 37°C (An overnight RNase treatment helped achieving in proper genomic DNA).

5. The dissolved DNA was extracted with equal volumes of phenol : chloroform: Isoamylalcohol (25:24:1, v/v/v) at 8000 rpm for 15 min.

6. The aqueous layer was transferred to a fresh 15 ml tube and reextracted with equal volume of chloroform and Isoamylalcohol (24:1) by centrifuging at 12,000 rpm for 15 min.

7. The supernatant was transferred to a fresh tube and equal volumes of absolute alcohol and 1/10 volume of sodium acetate were added and incubated at -20°C for 30 min followed by centrifugation at 12,000 rpm for 15 min. The pellet was air dried and resuspended in TE buffer. All the centrifugation steps were carried out at RT to avoid precipitation with CTAB, DNA degradation and to obtain good quality DNA.

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a UV Spectrophotometer (Cintra 5, GBC Scientific, Australia) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8% agarose gel based on the intensities of band when compared with the Lambda DNA marker (used to determine the concentration). The nucleic acid concentration was calculated following Sambrook et al. (1989).

Optimization of RAPD reaction

For the optimization of RAPD reaction using DNA extracted from various plant species, oligonucleotide primers from A and C series (Operon Technologies Inc. Alameda CA, USA) were used for amplification to standardize the PCR conditions.

The reactions were carried out in a DNA Thermocycler (MJ Research Inc. USA). Reactions without DNA were used as negative controls. Each 15 µl reaction volume contained about 50 ng of template DNA, 1X PCR Buffer (10 mM Tris Hcl pH 8.3; 50 mM KCl), 3 mM MgCl₂ (Invitrogen Life Technologies, Brazil), 0.2 mM dNTP Mix (Genetix, New Delhi, India), 0.5 µM of single primer (Genetix, New Delhi, India), 0.2 U of *Taq* DNA polymerase (Invitrogen Life technologies, Brazil). The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 37°C, extension was carried out at 72°C for 1 min and final extension at 72°C for 7 min and a hold temperature of 4°C at the end. PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TBE Buffer at 50 V for 3 h and then stained with ethidiumbromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Lambda DNA

Table 1. Optimization of the RAPD-PCR reaction parameters for 1. *Vitex pubescens*, 2. *Nervilia aragoana* 3. *Gymnema sylvestre* 4. *Withania somnifera* 5. *Origanum majorana*, 6. *Boswellia serrata* 7. *Saraca asoca* and 8. *Gloriosa superba*, medicinal and aromatic plants.

PCR Parameter	Tested range	Optimum conditions	Remarks
DNA concentration (ng)	5, 10, 20, 30, 40, 50, 75, 100, 150, 175 and 200	50 ng	Absence of amplification with lower concentration and presence of smear at higher concentration effected the repeatability.
Magnesium chloride (mM)	1, 2, 3, 4, and 5	3 mM	Excess/lower concentration increases the non specificity and yield of the product.
Deoxynucleotide triphosphates (dNTPs) (mM)	0.1, 0.2, 0.3, 0.4, 0.5	0.2 mM	Increased concentration reduces the free Mg ²⁺ , interfering with the enzyme.
Primer concentration (μM)	0.1, 0.5, 1, 1.5, 2, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0	0.5 μM	Lower and higher concentrations lead to absence of amplification and primer dimer formation, respectively.
<i>Taq</i> polymerase (units)	0.1, 0.5, 1.0	0.2 U	Lower concentration did not show proper amplification. High concentration showed decreased specificity.
Initial denaturation time interval (min) at 94°C	2, 3, 4 and 5	94°C for 3.0 min	Higher/lower time intervals (from optimum) leads to reduction in amplification, loss of <i>Taq</i> polymerase activity and lack of reproducibility
Annealing temperature (°C)/ Time intervals (s)	25, 30, 35, 37, 45, 50, 55, 60, 65 and 70 30, 60, 90 and 120	37°C for 60 s	Higher/lower annealing temperatures (from optimum) results in difference in specificity
Reaction volume (μL)	15, 25, 50	15	Influences the cost of the PCR ingredients
Number of cycles	25, 30, 35, 40 and 50	30	Higher/lower cycles (from optimum) effects the amplification

EcoR1- *Hind111* double digest was used as molecular marker (Bangalore Genei, Bangalore, India) to know the size of the fragments.

RESULTS AND DISCUSSION

DNA extraction was improved by modifying some of the steps in the original CTAB DNA isolation protocol (Doyle and Doyle, 1987). Leaves should be fresh and young. Samples frozen in liquid nitrogen and stored at -80°C for several weeks can also be used. Presented procedure resulted in extracting, high quality, low-polysaccharide genomic DNA from eight different plant species belonging to different genera including recalcitrant woody species.

The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications (Katterman and Shattuck, 1983; Peterson et al., 1997; Porebski et al., 1997). Tannins, terpenes and resins considered as secondary metabolites are also

difficult to separate from DNA (Ziegenhagen and Scholz, 1998). Certain polysaccharides are known to inhibit RAPD reactions. They distort the results in many analytical applications and therefore lead to wrong interpretations (Kotchoni et al., 2003). Polysaccharides like contaminants, which are undetectable by most criteria, can cause anomalous reassociation kinetics.

Polysaccharide co-precipitation is avoided by adding a selective precipitant of nucleic acids, i.e. cetyltrimethylammonium bromide (CTAB) to keep polysaccharides in solution though SDS (Dellaporta et al., 1983), sorbitol and glucose were added initially in the extraction buffer, which did not exhibit any effect. Addition of PVP along with CTAB may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent. Long-term chloroform : isoamylalcohol treatment ensured removal of chlorophyll and other colouring substances such as pigments, dyes, etc. Many DNA isolation procedures also yield large amounts of RNA, especially 18S and 25S rRNA (Doyle and Doyle, 1987; Mejjad et al., 1994; Rogers and Bendich, 1985). Large amounts of RNA in the sample can chelate Mg²⁺ and reduce the yield

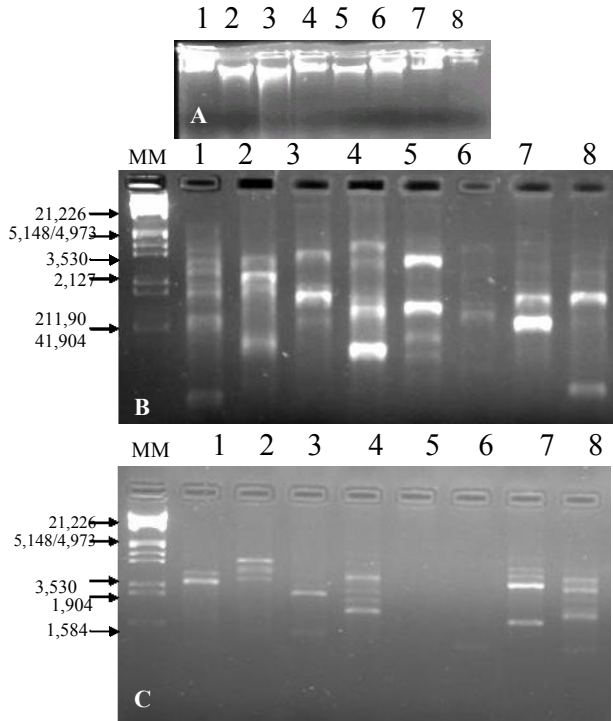


Figure 1. a) Lanes 1-8 represents the agarose gel (0.8%) of DNA isolated from 8 different plant species viz. 1. *Vitex pubescens*, 2. *Nervilia aragoana* 3. *Gymnema sylvestre* 4. *Withania somnifera* 5. *Origanum majorana*, 6. *Boswellia serrata* 7. *Saraca asoca* and 8. *Gloriosa superba*. **b)** Representative gel fractionation of RAPD-PCR amplification products of plant genomic DNA (50 ng). Amplification products were fractionated in a 2% agarose gel. Lane MM represents molecular marker (Lambda DNA *Hind*III/*Eco*RI double digest) Lanes 1-8 represents the RAPD- PCR products amplified from genomic DNA of eight plants as indicated in figure 1a using the Operon primer OPA-5 (5'-AGGGGTCTTG-3'). **c)** Represents gel fraction of RAPD-PCR with OPA-8 (5'-GTGACGTAGG-3') for the chosen eight plants as mentioned in figure 1a.

of the PCR. A prolonged overnight RNase treatment degraded RNA into small ribonucleosides that do not contaminate the DNA preparation, and yielded RNA-free pure DNA.

Additional precipitation steps removed large amounts of precipitates (detergents, proteins and polysaccharides)

by centrifugation and modified speed and time. DNA degradation and precipitations were avoided to some extent by carrying out all the steps at RT. We found these modified steps necessary to standardize and increase the quality and quantity of genomic DNA. The degree of purity and quantity varies between applications (Zidani et al., 2005). The extracted DNA was of high quality as it showed a reading of between 1.6 to 1.7 after calculating the 260/280 nm absorbance (Figure 1a). The DNA yield obtained ranged from 1 to 2 µg/µl.

DNA isolated by this method yielded strong and reliable amplification products showing its compatibility for RAPD-PCR using random decamer primers (Figures 1b and c, Figures 2a and b). Almost all the tested parameters for RAPDs like the concentration of template DNA, primer, magnesium chloride, *Taq* polymerase, dNTPs and temperature and time intervals during denaturation, annealing and elongation were also optimized which also had an effect on amplification, banding patterns and reproducibility. The optimized conditions for RAPD protocol are given in Table 1. The size of the amplified fragments ranged from 831-4,268 bp.

The conditions described in the present work, modified for use in RAPD analysis, consistently amplified DNA fragments of plant species belonging to different genera with various medicinal and aromatic properties, which are highly recalcitrant. The present optimized protocol for DNA isolation and RAPD technique may serve as an efficient tool for further molecular studies.

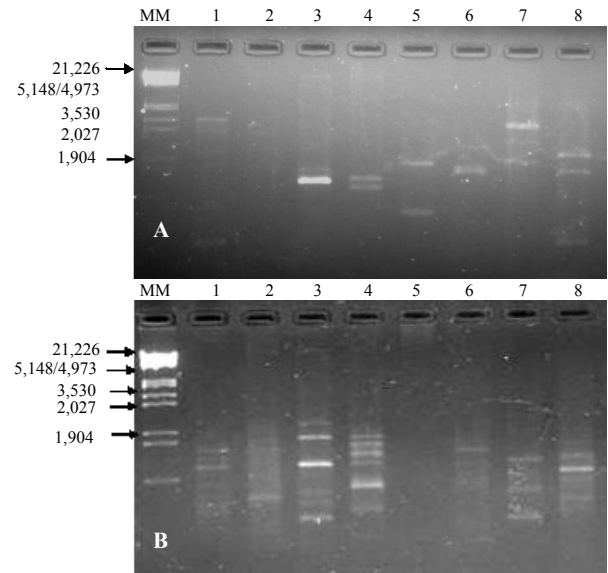


Figure 2. a) Representative gel fractionation of RAPD-PCR amplification products of plant genomic DNA (50 ng). Amplification products were fractionated in a 2% agarose gel. Lane MM represents molecular marker (Lambda DNA *Hind*III/*Eco*RI double digest) Lanes 1-8 represents the RAPD- PCR products amplified from genomic DNA of eight plants (for names refer figure 1) using the Operon primer OPC -10 (5'-TGTCTGGGTG-3'). **b)** Represents gel fraction of RAPD-PCR with OPC-15 (5'-GACGGATCAG-3').

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