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Optimization of DNA Isolation and PCR Protocol for ISSR Analysis of *Nothapodytes nimmoniana*: A Threatened Anti-cancerous Medicinal Plant

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ABSTRACT

Extraction of good quality DNA from *Nothapodytes nimmoniana,* an important anti cancerous medicinal plant containing many secondary metabolites, was a challenging task as many of these compounds co-precipitate with DNA and render the DNA unsuitable for downstream applications. Previously reported protocols yielded low quality brownish DNA, that was not amenable to further analysis. In this study, protocol was optimized with cetyltrimethylammonium bromide (CTAB) for isolation of genomic DNA. Important modifications in the optimized protocol included composition of homogenization buffer, volume of tissue: buffer ratio, modification of both extraction and precipitation temperature and duration. The efficiency and reliability of the method was tested by assessing quantity and quality of the extracted DNA by restriction analysis and Polymerase Chain Reaction (PCR) using Inter-Simple Sequence Repeat (ISSR). The yield of DNA ranged from 10-250 μg g^{-1} of leaf tissue. The PCR protocol comprising 50 ng template DNA, 0.5 mM dNTPs, 0.5 U Taq DNA polymerase and 0.5 μM primer concentrations found optimum for carrying out ISSR analysis in *N. nimmoniana*. The present optimized protocol for DNA isolation and ISSR technique serve as an efficient tool for carrying out further molecular studies in this species.

Key words: DNA extraction, *Nothapodytes nimmoniana*, ISSR, genomic DNA

INTRODUCTION

Nothapodytes nimmoniana Graham Syn. *Mappia foetida* is a threatened medicinal plant belonging to the family Icacinaceae. It is a rich source of anticancerous quinoline alkaloids camptothecin (CPT) and 9-methoxy camptothecin (9-mCPT) (Govindachari and Viswanathan, 1972). It is a medium sized tree distributed along the Western ghats of North East India, Sri Lanka, Myanmar and Thailand (Gowda *et al.*, 2002). CPT is a most promising anticancer drug of the twenty first century, which breaks single stranded DNA in mammalian systems (Hsiang *et al.*, 1985). CPT was found to be effective in the treatment of colon, head, breast, lungs and uterine cervical cancers (Takeuchi *et al.*, 1991; Potmesil, 1994). In addition to its antitumour property, CPT also possesses effectiveness against HIV, parasitic trypanosomes and *Leishmania* (Priel *et al.*, 1991; Satheesh Kumar and Seeni, 2000). It has been reported that *N. nimmoniana* also contain high amounts of alkaloids and essential oils such as camptothecin, 9-methoxy camptothecin, acetyl-camptothecin, hydroxy benzaldehyde, hydroxy propioguaiacone, scopoletin,

sitosterol, sitosteryl-β-D-glucoside, 3-β-hydroxy stigmast-5-en-7-one, 3-keto-octadec-cis-15-enoic acid, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (Wu *et al*., 1995; Hosamani and Pattanashettar, 2005).

The isolation of intact, high-molecular-weight genomic DNA is essential for many molecular biology applications. DNA is required for genetic mapping, cultivar identification, taxonomy, phylogenetic studies of animals and medicinal plants (Yasmin *et al*., 2006). DNA extraction from medicinal plants is a difficult task as they contain exceptionally high amounts of alkaloids, polyphenols, polysaccharides and essential oils. These compounds interfere in the extraction of good quality DNA and eventually inhibiting PCR, restriction digestion and other molecular biology applications. Degradation of DNA by endonucleases, inhibition of enzymes by polyphenols and tannins, co-isolation of polysaccharides, oxidation of secondary metabolites and co-precipitation of RNA are the important problems encountered during the extraction and purification of DNA from medicinal plants (Pikkart and Villeponteau, 1993; Weishing *et al*., 1995; Porebski *et al*., 1997).

However, there are many successful protocols to isolate nucleic acids from plant tissues with high contents of secondary metabolites, polyphenolics and polysaccharides. Some protocols adopted the use of reducing agents such as dithiothreitol and sodium metabisulfite while others successfully used the common CTAB associated with high salt as a reducing agent and selective precipitant of nucleic acids and polysaccharides (Dehestani and Tabar, 2007). Other methods like CD-C that involve modifications of the CTAB protocol have also proven successful in yielding high molecular weight DNA that is suitable for PCR applications (Srivastava *et al*., 2010).

We have tried previously reported CTAB and SDS based DNA extraction protocols (Doyle and Doyle, 1987) but all resulted in poor quality or denatured DNA. It was not suitable for restriction digestion and PCR amplification. To overcome the difficulties, we modified the reported DNA isolation protocol. The optimized CTAB based protocol resulted in the recovery of high quality and quantity DNA that is free from contaminants and coloured pigments and can be amplified by means of PCR.

MATERIALS AND METHODS

Plant materials: Fresh leaves of *N. nimmoniana* were collected from its natural habitat in Western Ghats of India. The tissue was brought to laboratory in an icebox and the DNA was isolated the following day.

Extraction buffer: 100 mM Tris HCl, 20 mM Na_2 EDTA, 1.4 M NaCl, 2% CTAB, 1.3% β-mercapto ethanol (added to buffer just before use). One gram Polyvinylpyrrolidone (PVP) per gram of tissue (added in the mortar while crushing the tissue).

DNA extraction protocol: One gram of leaf tissue was ground to a fine powder in a mortar and pestle using liquid nitrogen with 1 g PVP. The fine powder was quickly transferred to 15 mL (1:15 ratio) of freshly prepared prewarmed (65°C) extraction buffer and mixed vigorously by inversion to form slurry. The tubes containing slurry was incubated at 65°C for 60 to 90 min with intermittent shaking. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed vigorously by inversion for 25 times. The contents were centrifuged at 12,857 Xg for 15 min at room temperature to separate aqueous phase. The upper aqueous phase was transferred with wide bore pipette tip. An equal volume of chilled isopropanol was added to the aqueous phase and gently inverted the tubes several times to precipitate fibrous DNA followed by incubation at

-20°C for 1 h. The tubes were centrifuged at 12,857 Xg for 15 min at RT. The supernatant was discarded and DNA in pellet form was allowed to air-dry for 20-30 min. The DNA pellet was dissolved in 1 mL TE Buffer. Four microliter RNAse (10 mg mL^{-1}) was added and incubated at 37°C for 1 h.

Purification: Three milliliter TE and 4 mL chloroform: isoamyl alcohol (24:1) was added to the DNA sample, gently mixed and centrifuged for 15 min at 12,857 Xg at room temperature. Half volume of ammonium acetate (7.5 M) was added to the upper phase and mixed gently by inversion. Two volumes of saturated cold ethanol was added and kept at -20°C for 1 h. The contents were centrifuged at 12,857 Xg for 15 min at RT to pellet. The pellet was rinsed with 70% cold ethanol twice. The residual ethanol was allowed to evaporate by air-drying. The DNA was resuspended in 250 μL TE and incubated at 4°C for complete dissolution of DNA.

Quantity and purity of DNA: The DNA was quantified using UV-visible spectrophotometer at 260 nm and the purity was checked from $A_{260/280}$ ratio. To confirm the concentration and purity, the DNA was subjected to electrophoresis on 0.8% agarose gels prepared in 1X TAE with lambda DNA ladder.

Restriction endonuclease digestion: The isolated genomic DNA (1 μg) was digested with 10 units of restriction enzymes-Eco RI and Bam HI with 10X buffer (Fermentas) for 16 h at 37°C in 10 μL reaction volumes. The digested DNA was subjected to electrophoresis on 1% agarose gel and assayed by visual inspection on an UV transilluminator.

Optimization of ISSR PCR reactions and primer screening: Protocol for the PCR was optimized using varying concentrations of template DNA, dNTPs, Taq DNA polymerase and annealing temperature. One hundred ISSR primers purchased from the University of British Columbia (UBC set No. 9) were screened using a couple of DNA samples. The reactions were carried out in a DNA thermocycler (Eppendorf mastercycler gradient, Germany). Reactions without DNA were used as negative controls. Each 10 μL reaction volume contained varying concentration of template DNA (5-200 ng), dNTPs (0.1-1 mM) (Chromous Biotech, Bangalore, India), Taq DNA polymerase (0.1-0.3 U) (Chromous Biotech, Bangalore, India), ISSR primers (0.1-0.3 μM) and 1X PCR buffer (10 mM Tris HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$) (Merck). The thermocycler was programmed for an initial denaturation step of 94°C for 4 min, followed by 34 cycles at 94°C for 1 min, 45 sec at the specific annealing temperature of each primer (45 to 69°C) and 72°C for 1 min and a final extension at 72°C for 8 min and a hold temperature of 4°C at the end.

PCR products were electrophoresed on 1.5% agarose gel in 1X TAE buffer at 75 V for 2 h and then stained with ethidium bromide (0.5 μg mL^{-1}). Gel with amplification fragments were visualized and photographed by gel documentation system (Syngene). 1 kb molecular ladder was used as molecular marker to know the size of the fragments.

RESULTS

The standardized protocol yielded good quality genomic DNA. $A_{260/280}$ ratio values ranged from 1.7-2.01, which indicates insignificant levels of contaminating proteins and polyphenols.

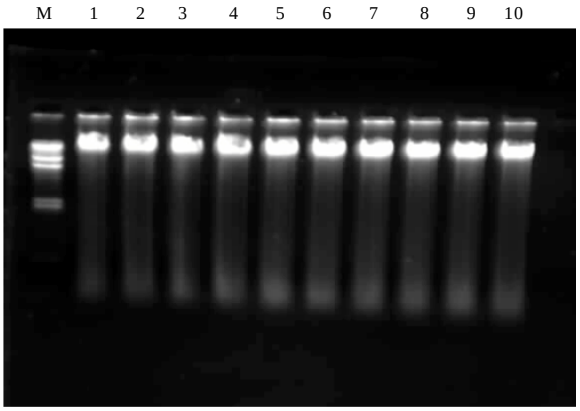

Fig. 1: Genomic DNA of *N. nimmoniana* isolated from the samples of Western ghats of India (Wayanad Dt. of Kerla)

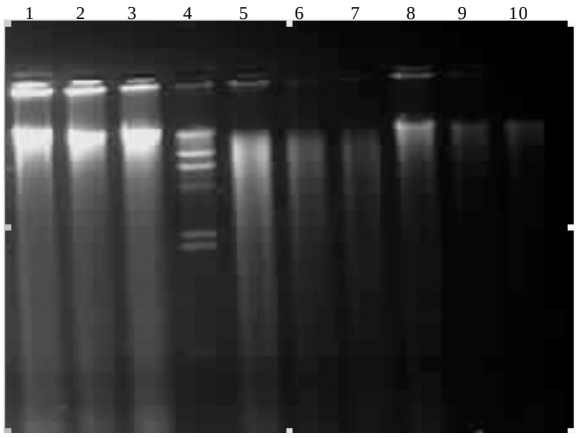

Fig. 2: Restriction digested DNA with Eco RI and Bam HI. Lane 1-3: genomic DNA, 4: Molecular weight ladder, 5-7: Restricted DNA with Eco RI, 8-10: Restricted DNA with Bam HI

Polyphenols and polysaccharides bind firmly to nucleic acids during DNA isolation and interfere with subsequent reaction (Dehestani and Tabar, 2007). The yield of DNA ranged from 10-250 μg^{-1} of leaf tissue. The agarose gel electrophoresis (0.8%) of genomic DNA showed high molecular weight bands of DNA (Fig. 1). The purity of DNA was confirmed through complete digestion of genomic DNA by restriction enzymes Eco RI and Bam HI (Fig. 2) and PCR amplification using ISSR primer (Fig. 3, 4).

Graded concentrations of dNTPs from 0.1 to 1 mM showed that 0.5 mM in the reaction mixture was optimum for generating reliable PCR products. Taq DNA polymerase at 0.1-0.3 U μL^{-1} tried. A final concentration of 0.15 U was found optimum for ISSR analysis. The primer concentration tested varied from 0.1-5 µM. The concentration of 0.5 µM was found optimum for amplification of *N. nimmoniana* DNA. Hence, 50 ng template DNA, 0.5 mM dNTPs, 0.5 U Taq DNA polymerase and 0.5 µM primer concentrations were optimum for carrying out ISSR analysis in *N. nimmoniana* (Table 1).

From the preliminary screening 29 ISSR primers showing amplification were selected for further examination. Different annealing temperatures were tested to optimize the amplification condition for the 29 selected primers. Eventually, 16 ISSR primers that produced clear and reproducible bands were selected for the amplification of all samples (Table 2). Reproducible amplifiable products were observed in all PCR reactions (Fig. 3, 4).

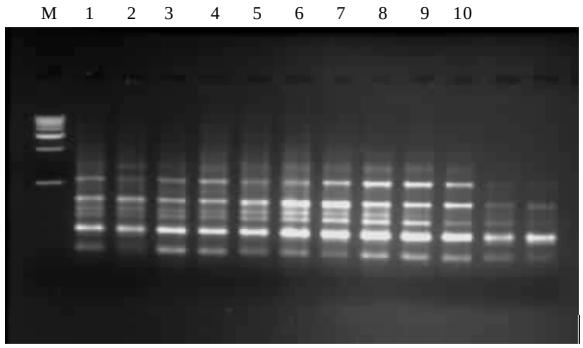

Fig. 3: ISSR profile of different accessions of *N. nimmoniana* with UBC 857 primer

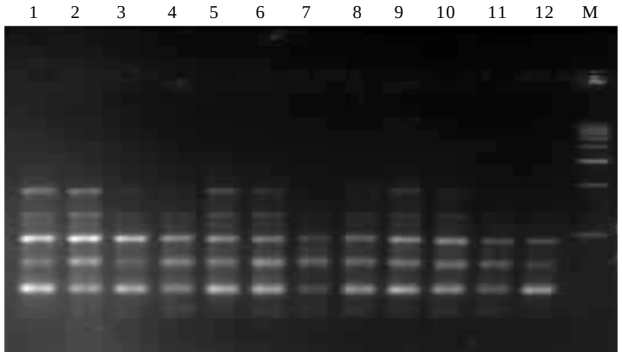

Fig. 4: ISSR profile of different accessions of *N. nimmoniana* with UBC 866 primer

Table 1: Optimization of the ISSR-PCR reaction parameters for *N. nimmoniana*

PCR parameter	Tested range	Optimum conditions	Remarks
DNA concentration (ng)	5, 10, 20, 30, 40, 50, 75,100, 150, 175 and 200	50	Absence of amplification with lower concentration and presence of smear at higher concentration
Deoxynucleotide triphosphates (dNTPs) (mM)	0.1, 0.2, 0.3,0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1	0.5	Increased concentration reduces the free Mg^{2+}, interfering with the enzyme
Primer concentration (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0	0.5	Lower and higher concentrations lead to absence of amplification and primer dimer formation, respectively
Taq polymerase (units)	0.1, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.2, 0.25, 0.3	0.15	Lower concentration did not show proper amplification. High concentration showed decreased specificity

Table 2: Details of screened ISSR primers with annealing temperature

UBC Primer Set # 9	Primer sequence (5'- 3')	Annealing temperature (°C)
810	GAG AGA GAG AGA GAG AT	45
844	CTC TCT CTC TCT CTC TRC	50
845	CTC TCT CTC TCT CTC TRG	53
847	CAC ACA CAC ACA CAC ARC	58
854	TCT CTC TCT CTC TCT CRG	50
857	ACA CAC ACA CAC ACA CYG	50
866	CTC CTC CTC CTC CTC CTC	55
873	GAC AGA CAG ACA GAC A	45
874	CCC TCC CTC CCT CCC T	55
886	VDV CTC TCT CTC TCT CT	58
890	VHV GTG TGT GTG TGT GT	53
891	HVH TGT GTG TGT GTG TG	48
895	AGA GTT GGT AGC TCT TGA TC	52
901	GAG CAA CAA CAA CAA CAA	53
905	AG AG AG AG AG AG AG AG	55
906	CCA CCA CCA CCA CCA	58

DISCUSSION

The significant obstacles in recovering good quality DNA were the oxidation of secondary metabolites and its co-precipitation with DNA. To overcome this problem, we have optimized the contents of homogenization buffer, volume of buffer, modification of both extraction and precipitation temperature and duration.

The tender leaves were not suitable for pure DNA isolation, which resulted in brown colored DNA. It may be due to the presence of high levels of CPT-the alkaloid. Padmanabha *et al.* (2006) found that CPT content in the leaves of seedlings is high as that in the wood, the CPT is the rich part of the plant. The mature and half mature leaves contain very little CPT content as compared to other plant parts. So the suitable stage of leaves for good quality DNA extraction is the fully developed light green coloured leaves rather than very tender leaves. The comparison of genomic DNA yield from five medicinal plants and their different organs (leaves, nodal stem and meristematic region) recorded good yield of DNA from leaves, nodal stem and meristematic region (Tiwari *et al.*, 2012).

The homogenization buffer contained higher β-mercapto ethanol. Increased amount of β-mercaptoethanol (1.3%) helped to check the oxidation of phenolic compounds and avoided its subsequent covalent bonding with DNA. One gram PVP per gram leaf tissue helped in the removal of phenolics and other impurities. 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. Also in addition of Poly Vinyl Pyrrolidone (PVP) in CTAB protocol, increased the solubility of polysaccharides present in woody plants (Sahasrabudhe and Deodhar, 2010). Increasing the volume of tissue, extraction buffer ratio from 1:10 to 1:15 helped to dissolve the phenolic contents and other secondary metabolites and later eliminated by centrifugation.

All the centrifugations were performed at room temperature so it prevented the co-precipitation of impurities and CTAB. Extraction with phenol:chloroform:isoamyl alcohol (25:24:1) ensured the selective removal of proteins and colouring substances such as pigments, dyes etc. The first extraction with chloroform:isoamyl alcohol resulted in brown coloured supernatant but the use of phenol:chloroform:isoamyl alcohol resulted in colourless supernatant. Addition of RNAse A and incubation at 37°C for longer duration (1 h) ensured the complete removal of RNA contaminants.

Two stages precipitation with isopropanol and ethanol reduced the presence of impurities. The precipitation time and temperature were also standardized as at -20°C for 1 h which reduced the co precipitation of contaminants to some extent. Similarly Gurudeeban *et al.* (2011) reported a modified cetyltrimethylammonium bromide extraction, using polyvinyl pyrrolidone while grinding, successive long-term chloroform:isoamy alcohol extractions, an overnight RNAse treatment with all steps carried out at room temperature.

The final DNA pellet was colourless and completely dissolved in TE, which indicated the absence of any other contaminants. DNA purity and quantification, Eco RI-restricted total genomic DNA and RAPD analyses showed differences in the efficiency and applicability of the extraction method, even within the same clonal cultivar (Da Silva, 2005). Srivastava *et al.* (2010) described two methodologies that yielded good-quality, high-molecular weight DNA that is free of contaminants and colored pigments and is suitable for PCR amplification in *Aconitum heterophyllum* Wall-endangered medicinal Species.

ISSR markers are valuable for determining interspecific variation and molecular characterization in *Utricularia* (Rahman, 2007). DNA concentration, primer sequence, primer concentration and annealing temperature are the critical parameters for obtaining reproducible banding patterns. Template DNA was the most important parameter to be optimized. The graded levels of DNA (5-200 ng) were used for determining the optimum concentration for ISSR analysis. Our protocol consistently produced a good yield of high quality DNA. The present optimized protocol for DNA isolation and ISSR technique may serve as an efficient tool for carrying out further molecular studies in this species.

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