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COMPARATIVE STUDY OF DIFFERENT GENOMIC DNA EXTRACTION PROTOCOL FOR *MURRAYA KOENIGII* (L.) SPRENG

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The present study was undertaken in curry leaf plant (*Murraya koenigii*), one of the most important aromatic and medical plants in the world to explore the best DNA isolation protocol. Presence of abundant quantity of secondary metabolites in curry leaf plant resulted in poor quality and quantity of isolated genomic DNA. Thus, two DNA extraction protocols namely CTAB (Cetyl Trimethyl Ammonium bromide) modified method and Dellaporta modified method were evaluated for extraction of DNA. The quantity and quality of the DNA extracted was compared using UV-spectrophotometer and agarose gel electrophoresis. CTAB modified method was found to exhibit consistently positive results in terms of both quality and quantity of DNA (A260/280) as compared to Dellaporta method. The isolated DNA was highly intact and devoid of shearing. Further, this method was explored to different species of Rutaceae family, giving significant DNA yield.

Keywords: *Murraya koenigii;* DNA extraction; Genomic DNA; CTAB; Rutaceae.

Introduction

Curry leaves are the leaves of the curry tree, scientifically known as *Murraya koenigii* (L.) Spreng and it belongs to the Rutaceae family. The plant is native to India and is usually found in tropical and subtropical regions. *Murraya koenigii* is widely used in Indian cookery from centuries and have a versatile role to play in traditional medicine. The useful parts of this plant are its leaves, roots, and its bark. Curry leaves are natural flavoring agents with a number of important health benefits, which make your food both healthy and tasty along with giving it a pleasant aroma. They are also believed to have cancer-fighting properties and are known to protect the liver.

The plant is credited with tonic and stomachic properties. Bark and roots are used as stimulant and externally to cure eruptions and bites of poisonous animals. Green leaves are eaten raw for cure of dysentery, diarrhoea and vomiting. Leaves and roots are also used traditionally as bitter, anthelmintic, analgesic, curing piles, inflammation, itching and are useful in leucoderma and blood disorders[1,2]. This plant has been reported to have anti-oxidative, cytotoxic, antimicrobial, antibacterial, anti ulcer,

positive inotropic and cholesterol reducing activities[3-7].

Molecular techniques are very useful for exploiting genetic diversity and also for authentication and identification of genotypes. Now-a-days, molecular aspects of biological studies are highly valuated and the first approach to such fields is extraction of nucleic acids. Depending on the nature and complexity of plant material, the isolation of high purity genomic DNA is an important pre-requisite to achieve a logical conclusion for molecular biological applications. The extraction of high-quality DNA is important in any molecular biology work because contaminants such as proteins, polyphenols, and polysaccharides may interfere with enzymes such Taq polymerase in polymerase chain reaction[8, 9]. Here, we optimized and evaluate the quality and quantity of genomic DNA isolation protocol suitable for *Murraya koenigii* covering *in vitro* grown plantlets to relatively complex plant tissues such as field grown mature plant .

Materials and Method

(i) Plant Materials:

Plant material for genomic DNA isolation was collected from a mother plant (ten years old field-grown plant) from surrounding areas of Ajmer in the month of March to the end of July and micropropagated plants developed through different types of explants such as nodal & inter-nodal segments of matured tree and epicotyl, cotyledons, cotyledonary node (embryonic axis), juvenile leaf, hypocotyl and root segments from 60 days old *in vitro* raised seedlings of *Murraya koenigii* [10].

In vitro shoots were established from different types of explants were maintained in optimized media for 5 cycles. Subsequent sub - cultures were done at a fixed frequency of 4 weeks on fresh medium.

(ii) DNA Extraction:

Young & juvenile leaf tissue of micropropagated plantlets as well as the mother plant was used for molecular analysis. The juvenile leaves of field grown and micropropagated plants were used for extraction of genomic DNA by CTAB and Dellaporta methods.

(a) Dellaporta method

Genomic DNA was extracted by following the protocol described by Dellaporta *et al.* (1983)[11]. The young & juvenile leaf tissue (1 g) was ground in liquid nitrogen to fine powder. The powder was transferred, preventing thawing, to 15 ml. DNA extraction buffer containing 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 10 mM β–mercaptoethanol (added freshly). To this solution, 500 µl 20% SDS was added and after thorough mixing, the solution was incubated at 65°C in water-bath. This was followed by addition of 2.5 ml potassium acetate (5M) and incubation in ice for 20 min. Subsequently, the reaction mixture was centrifuged at 14,000 rpm and supernatant was filtered through muslin cloth. DNA was precipitated using 0.6 volume of chilled isopropanol and pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was suspended in high salt TE (50 mM Tris-Cl (pH 8.0) and 10 mM EDTA) and again centrifuged at 10,000 rpm to remove any debris present. DNA was again precipitated and resuspended in 1X TE (10 mM Tris-Cl (pH 8.0) and 1 mM EDTA).

(b) CTAB method

Genomic DNA was also extracted following the Cetyl Trimethyl Ammonium bromide (CTAB) protocol described by Saghai-Maroof *et al.* (1984)[12] with minor modifications. Leaf tissue (1 g) was ground to fine powder in liquid nitrogen and transferred to 10 ml 2X CTAB buffer

containing 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB (w/v) and 10 mM β–mercaptoethanol (added freshly). It was mixed vigorously by vortexing and incubated at 60°C for 30 min followed by treatment with equal volume of chloroform: isoamyl alcohol (24: 1). The upper phase, obtained by centrifugation at 10,000 rpm for 15 min at room temperature, was transferred to a fresh autoclaved centrifuge tube and to it was added 1/10 volume 3M sodium acetate (pH 5.2) and 1/2 volume 5M NaCl. DNA was precipitated using 0.6 volume chilled isopropanol and pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was decanted and the DNA pellet will be washed twice with 70% ethanol. DNA pellet was air dried and suspended in 500 µl of 1X TE

(iii) DNA Purification:

Since the DNA contained RNA as impurity, the same was removed by RNAase treatment[11]. Bovine Pancreatic RNAase (5 mg/ml) was added (100 µg RNAase/ml DNA suspension) to the dissolved DNA and kept for incubation at 37°C for one hour. To remove the RNA and other proteins as impurities, an equal volume of chloroform: isoamyl alcohol (24:1) was added to the DNA solution, mixed by inversion and centrifuged at 10,000 rpm for 15 min at 4°C. The upper aqueous phase was pipetted out in another MCT using wide bore pipette tips. To it was added $1/10^{th}$ volume sodium acetate (3M) and equal volume of chilled isopropanol and kept at –20°C for one hour. DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C, washed with 70% ethanol; air dried and dissolved in 100 µl 1 X TE buffer.

(iv) DNA Quantification:

DNA concentration was estimated using spectrophotometric method (UV-Vis Spectrophotometer, Biotech. Engineering Management Co., U.K). 5µl DNA sample was added in 3000µl MQ water in cuvette. Absorbance of the solution was measured at wavelengths 260 nm and 280 nm [13]. The DNA concentrations were calculated using following formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{50 \times OD_{260} \times \text{Dilution Factor}}{1000}$$

The ratio of OD_{260}: OD_{280} was also calculated. The same procedure was followed for quantifying all the samples. DNA samples were diluted to final concentration of 10ng/µl for use in PCR analysis. DNA samples which had best quality as evident on agarose gel and an OD_{260}/OD_{280} ratio nearer 1.8 to 2.0 were used for further analysis.

Genomic DNA (1µg) was digested overnight with 10 units of each of the three restriction enzymes such as *Eco* RI, *Hind* III and *Bam* HI individually. The reaction was carried out in buffered condition at 37°C following manufacturer's instructions (Bangalore Genei, Bangalore, India). The three digested genomic DNA was electrophoresis on 0.8 % agarose gel in order to confirm the purity genomic DNA.

(v) Agarose Gel Electrophoresis:

The quality of DNA was checked by Agarose (Bio-Rad & Hi-media) Gel Electrophoresis. For visualization of genomic DNA 0.8% and for amplified products 1.5% agarose gels were prepared. Agarose powder was added to 1 X TAE buffer and boiled in microwave oven. This solution was cooled to 45–50°C under running tap water. To it added 0.5 µg/ml ethidium bromide was added, shaken gently and poured into gel casting tray having pre–adjusted comb. The gel was allowed to set at room temperature.

The comb was carefully removed

and the gel casting tray was placed in submarine gel tank. The gel tank was then filled with 1 X TAE buffer ensuring that the gel was completely submerged in the buffer. Samples were prepared by taking 4.0 µl of DNA and 1.0 µl of 10 X bromophenol blue dye (0.25% Bromophenol Blue and 50% glycerol) on a Parafilm^{TM} strip and mixed well with the help of a micropipette. PCR products were electrophoresed in 0.5 X TBE buffer for ca. 1 h at 80 V on a 1.5% agarose gel matrix. All the amplified products were separated on 1.5% agarose (w/v) gel, stained with 0.5 µg/ml ethidium bromide. DNA ladder 1 Kb (Bangalore Genei, India) and 100 bp DNA ladder (Bangalore Genei, India) were mixed and used as molecular weight marker for comparison of amplified products. Gels were photographed under UV light using a Gel Documentation System "Biovis" (Expert Vision, Mumbai, India). All reactions were repeated thrice to confirm the results.

Results and Discussion

(i) Quantification of Genomic DNA:

A comparative study of DNA quantities extracted from different samples by Dellaporta and CTAB methods was also undertaken. The quantity of DNA was varied in different samples and methods used. The quantity of DNA per microlitre from all the samples extracted from CTAB and Dellaporta methods is shown in Fig.1.

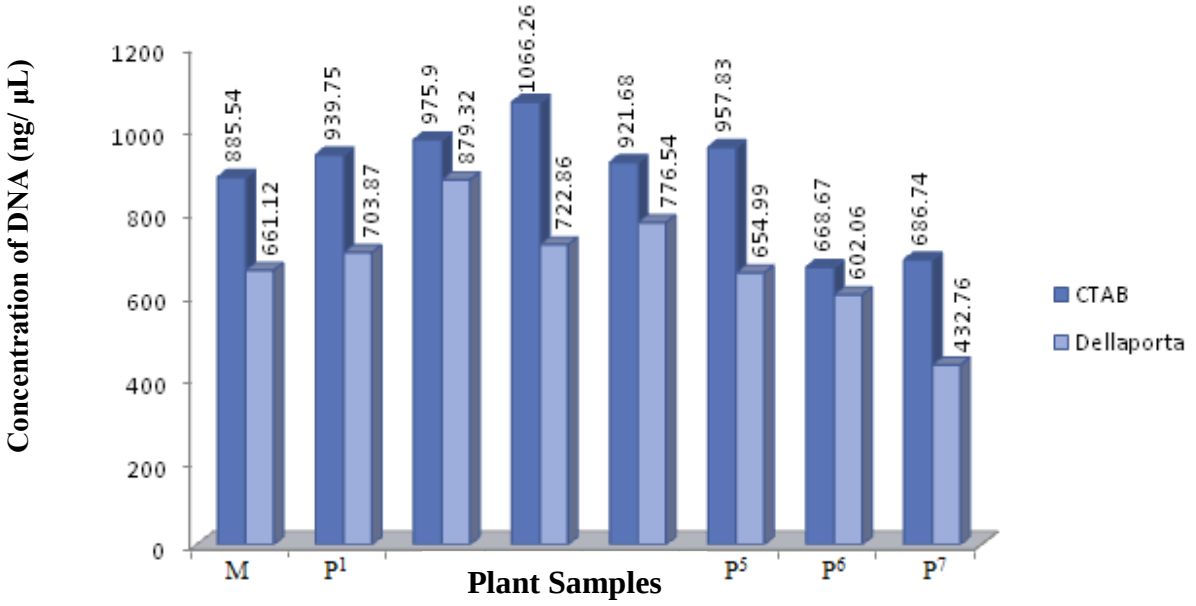

Fig. 1 Spectrophotometric Quantification of Genomic DNA of *Murraya koenigii* **(M** parent plant and micro-clones developed through **P**[1] Inter-nodal (IND), **P**[2] Hypocotyl (HYP), **P**[3] Root (RT), **P**[4] Cotyledon (COT), **P**[5] Cotyledonary Node (CN), **P**[6] Epicotyl (EP), **P**[7] Nodal (ND**)** segments

For checking the yield and the presence of dissolved impurities, the genomic DNA samples were used in spectrophotometric analysis for measuring absorbance at 260 nm and 280 nm, respectively. Spectrophotometric analysis at A260/A230 revealed ratio ranged from 1.71 to 1.96 (Table 1.).

Table 1. Quality of genomic DNA extracted from leaves of regenerates and mother plant of *Murraya koenigii* using CTAB & Dellaporta extraction methods.

Well no.	DNA Samples	Absorption Ratio (260nm/280nm)	
		Dellaporta method	CTAB method
H1	Nodal (ND)	1.75	1.84
H2	Inter-node (IN)	1.83	1.89
H3	Epicotyl (EP)	1.79	1.85
H4	Leaf (L)	1.80	1.88
H5	Cotyledons (COT)	1.84	1.92
H6	Cotyledonary node (CN)	1.88	1.96
H7	Hypocotyl (HYP)	1.71	1.81
H8	Root segment (RT)	1.76	1.82

CTAB modified method was found to exhibit consistently positive results in terms of both quality and quantity of DNA (A260/280) as compared to Dellaporta method. The isolated DNA was highly intact and devoid of shearing (Fig. 2.)

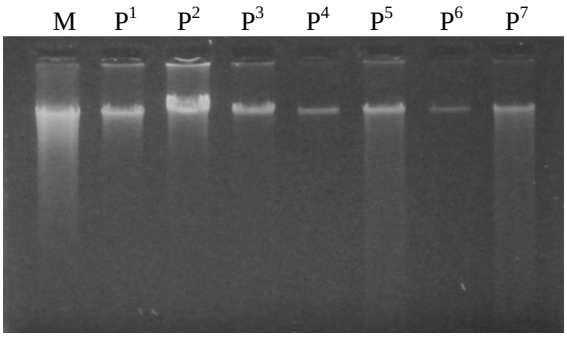

Fig. 2. Electrophoresis on agarose gel for DNA extracted from CTAB method (**M** parent plant and micro-clones developed through **P**[1] Inter-nodal (IND), **P**[2] Hypocotyl (HYP), **P**[3] Root (RT), **P**[4] Cotyledon (COT), **P**[5] Cotyledonary Node (CN), **P**[6] Epicotyl (EP), **P**[7] Nodal segments (ND**)** segments

(ii) Evaluation of DNA Samples for PCR Amplification:

Total Genomic DNA Extracted from two different methods was tested for PCR amplification (Bio-Rad, USA) under similar conditions. Comparisons between amplification products obtained by different methods were made on 1.5 % agarose gel. RAPD profiles were produced through PCR amplification using the protocol described by Verma & Rana (2012)[14]. PCR amplification was performed in 25 µl reaction volumes containing 2.5 µl of 10X assay buffer. All the reagents of PCR amplification reaction mixtures used were procured from *Invitrogen Bioservices*, Bangaluru, India.

The amplification reaction was carried out in a DNA Thermal Cycler (T100, Bio-Rad, USA). The quantities of thermal cycler reaction mixtures are shown in Table-2 and PCR programme involved an initial denaturation at 94°C for 01 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 32°C, 2 min primer extension at 72°C and final extension for 5 min at 72°C. After completion of the PCR amplification, 5.0 µl of the amplified products were electrophoresed in a 1.5% (m/v) agarose gels (Bangalore Genei, Bangalore, India) with 1X TAE buffer, stained with ethidium bromide, and were later photographed and documented by a gel documentation system. The sizes of the amplification products were

Table 2. Optimized reaction mixture for PCR amplification by RAPD primers.

S. No.	Component	Concentration
1.	Template DNA	50 ng
2.	PCR assay buffer	1X
3.	$MgCl_2$	2.5 mM
4.	dNTPs (dATP, dGTP, dCTP and dTTP)	300 μM
5.	Taq DNA Polymerase	1.5U
6.	Random Decamer Primer (OPU 17)	20 pmol

estimated by comparing them to a standard DNA ladder. All the reactions were repeated three times.

The CTAB protocol followed by purification yielded good quality DNA that was amenable to PCR amplification in comparison with Dellaporta protocol. More amplicons and prominent bands were observed in the CTAB samples (Fig. 3 A, B).

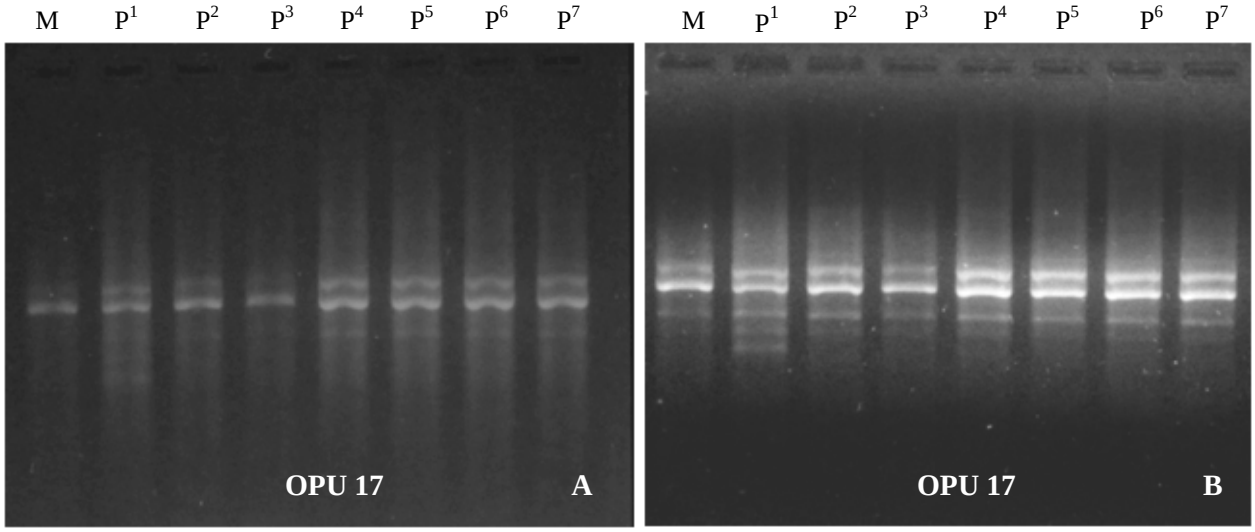

Fig. 3 (A, B) Comparative evaluation of PCR amplification of DNA extracted from leaves of *Murraya koenigii* using (**A)** Dellaporta method and **(B)** CTAB method (**M** parent plant and micro-clones developed through **P**[1] Inter-nodal (IND), **P**[2] Hypocotyl (HYP), **P**[3] Root (RT), **P**[4] Cotyledon (COT), **P**[5] Cotyledonary Node (CN), **P**[6] Epicotyl (EP), **P**[7] Nodal segments (ND**)** segments

Presence of some inhibitors in extracted DNA solution causes a reduction in Taq-polymerase activities in PCR and hinders the function of cutter enzymes as well[15]. Denaturation and removal of protein are very important to avoid its interference with DNA. Thus chloroform: isoamylalcohol (24:1) was used for denaturing proteins from DNA allowing only DNA in the supernatant after centrifugation step[16].

The major issue is that the polyphenols bind covalently with the isolated DNA and reduce its purity and yield[17,18]. The addition of Polyvinylpolypyrrolidone (PVPP)

(or its water-soluble counterpart polyvinylpyrrolidone (PVP) has been used to extract genomic DNA from other polyphenol-rich plants such as cotton, sugarcane, lettuce and strawberry[19], and grape, apple, pear, persimmon and several conifers[20]. PVP forms complex with latex lactones, actucin and other phenolics. The PVP complexes accumulate at the interface between the organic and the aqueous phases by centifugation after addition of chloroform. CTAB binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction[21, 22]. The genomic DNA of *Murraya koenigii* extracted by CTAB protocol in our laboratory was then subjected to RAPD analysis; the primer produced clear, scorable and reproducible bands this indicated that the isolated DNA was suitable for molecular biological applications like genetic fidelity.

Conclusion

The low cost, high throughput, high quality, high yield, and broad applicability of the method make it a useful method for PCR application that needs large quantity and high quality DNA of *M. koenigii*. The protocol reported in present research may meet the raw material demands of pharmaceutical industries for isolation of target compounds from *M. koenigii*.

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