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An Effective, Improved and Efficient Method of DNA Extraction from The New and Old Leaves of Mango (*Mangifera indica* L.)

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Abstract
Mango (*Mangifera indica* L.) is favoured for its delicious and juicy nature of the fruit and one of the important fruits of the world. It is commonly known as the "king of fruits". The present experiment was designed to develop a simple method to obtain good quality as well as a higher yield of DNA (Deoxyribonucleic Acid) from the leaves of ten mango cultivars at two different stages (new flush and old leaf). This modified extraction method of CTAB (Cetyl Trimethylammonium Bromide) has the ability to isolate the required quality of DNA from mango old leaves, which contains higher amounts of polysaccharides and polyphenols. This method of DNA extraction is useful when the young leaves are not available year-round. Extracted DNA samples of ten cultivars were used to PCR (Polymerase Chain Reaction) amplification by using SSR (Simple Sequence Repeat) primer Mng SSR-14 showed clearly visible DNA bands in new flush and old leaves.

Keywords: DNA extraction, DNA samples, Leaf stages, Mango, PCR amplification, Polyphenols

Introduction
Mango (*Mangifera indica* L.) is very popular for its attractive juiciness and pleasant aroma present in the pulp. It is designated as the "king of fruits" and commonly cultivated in tropical regions of the world, Singh (1996). It ranks first among all the fruits of India in area and production. Currently in India mango has cultivated in an area of 2.31 Million ha with a production of 22.35 Million Tonnes and 7.3 MT/ha productivity. Uttar Pradesh, Andhra Pradesh, Odisha, Karnataka, and Telangana are the major mango cultivating states in India. (NHB data base, 2019) [21].

In the present scenario, many of the cultivating mango varieties have synonyms in different regions, thus making it difficult for the identification of genuine cultivar. Morphological features are ineffective and inaccurate in differentiation and identification. Further, this problem is compounded by the monoembryonic and perennial nature of mango. The genetic improvement in mango can be improved by using genomic-based approaches. One of the major prerequisites for molecular studies is extracted DNA has enough good quality. Generally, all perennial crop leaves contain higher concentrations of polysaccharides, polyphenols, and other secondary metabolites, all enzymatic activities will be interrupted and changes occur in amplification of DNA in the presence of these compounds, Maria *et al.* (2001) [10]. The contaminated RNA along with DNA during the isolation procedure that interference with the amplification of PCR and poses many other problems like (Pikkart and Villeponteau, 1993; Padmalatha and Prasad, 2006) [13-14] improper priming of DNA templates during the sequencing of the thermal cycle in PCR.

Like other perennial woody crops, DNA isolation from mature leaves of mango is very difficult because of the presence of high phenolics, polysaccharides, and secondary metabolites these compounds interfere with DNA isolation procedure and inhibit the enzymatic activities during the isolation of DNA (Pirttilä *et al.* 2001) [15]. Good quality and high yield of DNA are obtained only from young leaves but these are not available throughout the year. So, the use of mature leaves would provide an alternative source of DNA extraction when sufficient new leaf tissue is not available. Some of the protocols related to the extraction of genomic DNA from young and fresh leaves of mango were reported in earlier studies (Davenport and Nunez-Elisea 1997; Davis *et al*. 1995; Dellaporta and Hicks 1983) [4-6],

but new flush does not get always on mango tree. Anyhow, vegetative growth is restricted to 3-4 times in a year, it depends upon the varieties and growing conditions (Davenport and Nunez-Elisea 1997) [4]. Good and high-quality pure DNA samples are required for the success of any further molecular studies. High intensity of DNA bands and low smear intensity were obtained in the electrophoresis is possible with high quality and uncontaminated DNA (Utami *et al.* 2012) [19]. Previously reported methods for DNA extraction such as Doyle and Doyle (1990) [7]. The use of a DNA isolation kit is cost effective even though these are becoming popular in genomic DNA extraction studies (Amani 2011) [1]. CTAB buffer method is most frequently used in DNA extraction (Pirttilä 2001) [15] plant that contains polysaccharides and polyphenolic compounds (Jose and Usha 2000) [9].

The main objective of the present experiment was to develop a simple procedure to get good quality and uncontaminated DNA with higher yield from mango leaves of two different stages (New leaf and old leaf). Optimized the DNA quantification with few alterations were focused on reducing the phenolic contents and polysaccharides for obtaining the good quality of DNA. CTAB method of DNA extraction explained in this study is a very simple, efficient, and effective method to get good quality DNA from new mango leaves.

Material and Methods

Plant material

Ten mango cultivars (*Mangifera indica* L.) i.e. Baneshan, Dilpasand, Himayath, Kaju, Mahamooda Vikarabad, Nazeem Pasand, Neeleshan, Neelum, Pulihora and Zardalu. These were collected from the Fruit Research Station, Sangareedy. The trees were tagged in the middle of June-July, 2019 at the time of new flush starts (7 days after the flush), simultaneously mature/old (50 days after new flush starts) leaf also collected from the same tagged trees for DNA extraction to see the difference between two leaf stages of mango. Five leaves of two different stages were collected from each variety. While selecting the leaf samples care was taken to avoid contaminations.

Collection and processing of leaf material

Five new and healthy leaves of each cultivar from a single tree were plucked and taken into labeled polythene covers and placed in an icebox until and unless reaching the laboratory and kept away from sunlight. Selected leaves were washed under running water, whipped with tissue paper. After removal of midribs and thick veins of leaves were wrapped in properly labelled aluminium foil, were dipped in liquid nitrogen to attain quick freezing and stored at –80 ºC till DNA isolation.

Chemicals and reagents

Chemicals used in the experiment include: CTAB 2 %, NaCl % 5 M, Ethylene Diamine Tetra Acetic acid (EDTA, 0.5 M) (Ethylenediaminetetraacetic Acid), Tris: HCl (Hydrochloric Acid) buffer (pH 8.0, 1 M), 2-Mercaptoethanol (2%), Poly venyl pyrrolidins (PVP) (Polyvinylpyrrolidone) 1% and 2 %, Chloroform: Isoamyl alcohol (24:1), Isoproponol, chilled ethanol 70 %, TE buffer (10X), DNA loading Dye (6X), 10× PCR buffer, 10 mmol/L dNTPs (Deoxyribonucleotide Triphosphate), and 50 mmol/L MgCl2 (Magnesium Chloride).

Genomic DNA extraction procedure

Genomic DNA was isolated by the cetylhexadecyl-trimethyl ammonium bromide (CTAB) method following the protocol of (Doyle and Doyle 1990) with few modifications in buffer composition and concentration.

Steps in DNA isolation from leaf tissues (new and old) of mango

- 100 mg of tender and older leaf samples of each variety were taken into a separate pestle and mortar, ground finely by adding 1000 µl of CTAB buffer.
- PVP 1 % and 2 % of prepared solutions were added to each sample of new leaf tissue and old leaf tissue respectively along with CTAB buffer while grinding.
- Immediately that grounded mixture transferred into 2 ml centrifuge tubes, vortexed to disperse the sample tissues in the mixture.
- These tubes were incubated in a water bath at 65°C for 1 hour, with intermittent shaking for every 10 minutes. After the incubation process, the tubes were cooled to normal temperature and 700 µl of chloroform: isoamyl alcohol (24:1) was added, and slowly mixed the contents by inverting the tubes for 10 minutes
- The contents were spin at 10,000 rpm for 15 minutes.
- Transferred the aqueous phase into a new tube (1.5 ml) and re-extracted with chloroform: isoamyl alcohol (24:1).
- The tubes were spin at 25°C for 15 minutes at 12,000 rpm. The supernatant aqueous phase which contains nucleic acids was taken out into new centrifuge tubes.
- Added an equal volume of chilled isopropanol to the aqueous phase and mix gently.
- To aid the precipitation, the tubes were spin at 10°C for 10 minutes at 10,000 rpm.
- The supernatant was discarded and the precipitated DNA pellet was washed twice with 70 percent ethanol and air dried the pellet until the ethanol smell has vanished
- The DNA pellet was dissolved in 100 ml $T_{10}E_1$ (Tris and EDTA) buffer and kept at -20°C for long term storage.

DNA purification and quantification

Purification of DNA was done by adding 2µl of RNase A (25 mg/ml) to the dissolved DNA were taken into a fresh tube and incubated for 1 hour at 37 °C. Thus, remove RNA, proteins and polysaccharides which were the major contaminants. The differentiation in the colour of isolated DNA pellets from new and old leaves of mango was seen visually.

Quantification and purity of ten cultivars DNA samples were checked by running in 0.8 % of agarose gel in 100ml of 1 X TBE (Tris-borate-EDTA) buffer and heat it in the microwave for 3 minutes. Upon cooling, Ethidium bromide was added and poured in a gel casting tray with a properly placed comb. Polymerization of the gel was allowed for 30 minutes, after which the comb was taken out carefully without any damage to the wells. The gel was transferred to an electrophoresis tank. Two µl of loading dye 1 X was added to each sample tube after completion of purification. The purified DNA samples were loaded into the gel wells with control. The concentration of DNA was determined by comparing the intensity of bands with that of 50 bp molecular ladder quality was indicated by having an intact band. The purity of DNA determined by estimating the ratio of absorbance at 260 nm to that at 280 nm (A260/A280).

PCR amplification

The isolated DNA from the listed genotypes was amplification was amplified by filling PCR reaction mixture (25 µl) contained 25 ng/µl DNA, 10× PCR buffer, 2.5 mM/µl dNTPs, 50 mM/µl MgCl2, and 10 pmole each of forward and reverse primers. The PCR mixture was set in a 96 welled microtiter plates and the program was configured in Eppendofr Master Cycler with an initial denaturation step of 4 min at 94 °C (initiation) followed by 35 cycles of 30 s at 94 °C (denaturation), 30 s at an appropriate annealing temperature (Primer Mng SSR-14; temperature 54.6 °C) and 1 min at 72 °C (extension). The PCR program closed with the final extension at 72 °C for 7min. The amplified PCR products were separated by electrophoresis on a 3% agarose gel (0.03 g/ml) containing ethidium bromide. Electrophoresis was carried out at 120v for 2-3 hours and then viewed under the Bio-Rad gel documentation unit.

Results and Discussion

For any molecular studies, proper selection of leaf tissue is one the prerequisite for getting good quality and pure DNA. According to Mauro (1992) [11] rapidly expanding leaves with one or two nodes from the shoot is the best material for the extraction of DNA. But new flush available only for 2-3 months but old leaves were throughout the year on the trees. Old and mature leaves are generally not considered because of thicker and tougher midribs and veins which makes grinding step difficult. According to Howland (1991) [8] that mature leaves contain higher concentrations of polyphenolic compounds.

Table 1: Composition of CTAB buffer

Component	Stock solution	Working buffer	Prepare 1000 ml buffer from stock	Prepare to 500 ml
Tris HCl	1 M	100 mM	100 ml	50 ml
NaCl	5 M	1.4 M	280 ml	140 ml
EDTA	0.5 M	20 mM	40 ml	20 ml
CTAB	2 %	2 %	20 g	10 g
PVP	1%	1 %	10 g	5 g
PVP	2 %	2 %	20 g	10 g
Mercaptoethanol	0.2	0.2%	2 ml	1 ml
D. H_2O	-	-	560 ml	280 ml

Table 2: DNA concentration levels and ratio of A260/280 and A260/230 in ten mango cultivars of new flush and old leaf tissues

Cultivar	New leaf			Old leaf		
	DNA ng/µl	A260/280	A260/230	DNA ng/µl	A260/280	A260/230
Baneshan	898.7	1.82	2.06	398.34	1.95	2.13
Dilpasand	1171	1.79	2.12	750.90	1.86	2.08
Himayath	879	1.69	2.01	495.7	1.78	2.10
Kaju	1493.7	1.72	2.21	604.1	1.92	2.11
Mahamooda Vikarabad	1185.3	1.89	2.26	744	1.93	2.28
Nazeem Pasand	738.6	1.65	2.05	495.6	1.76	2.14
Neeleshan	1023	1.69	2.11	981.8	1.73	2.25
Neelum	1078.2	1.86	2.24	619.1	1.97	2.29
Pulihora	1039.2	1.63	2.12	511.6	1.78	2.21
Zardalu	898.1	1.75	2.01	577.3	1.84	2.18

In the present experiment new flush (7 days old) (Figure 1a) was yielded white coloured, transparent and pure DNA rather than the old leaf (50 days) (Figure 1b). The present results were further supported by the fact that white coloured and transparent DNA pellet obtained from new leaf tissue ready to dissolved more rapidly in TE buffer compared to the yellowish and brownish pellet which is obtained from the old leaf tissues because of containing higher concentrations of polyphenolic compounds and polysaccharides

Concentration levels of ten cultivars DNA samples were checked through Spectrophotometer in new flush the recorded concentration was ranged from 879 ng/µl to 1493.7 ng/µl and in old leaves it was 981.8 ng/µl to 398.34 ng/µl (Table 2).

The DNA samples are contaminated with other molecules like proteins, organic compounds and other contents. The other benefit of utilizing spectrophotometric analysis at λ A260/280 nm (Sambrook *et al.*, 1989) [17] for quantification of DNA and the ability to estimate the sample purity. The ratio of A260/280 indicates the presence of molecules like proteins, phenolics, or other contaminants that absorb strongly at or near 280 nm. To check the purity level in nucleic acids was done by the ratio at 260/230 as a secondary measurement, the resulted values are mean to be “pure” the range of 260/230 is commonly between 2.0-2.2. Sometimes values are higher than the common range.

The ratio of A260/280 in new flush ranged from 1.63 (Pulihora) to 1.82 (Baneshan), while in old leaves it was 1.73 (Neeleshan) to 1.97 (Neelum). DNA concentration was higher in new leaves rather than old leaves. The ratio of A260/280 was low in the case of new flush, a lower ratio of A260/280 (~1.8) is considered that extracted DNA pure. This ratio was showed more in the case of old leaf. The ratio of A260/230 ranged from 2.01 (Himayath, Zardalu) to 2.26 (Mahamooda Vikarabad) observed in case of new flush, while in old leaves ratio was 2.08 (Himayath) to 2.29 (Neelum).

Fig: 1a and 1b two leaf stages of mango for DNA extraction

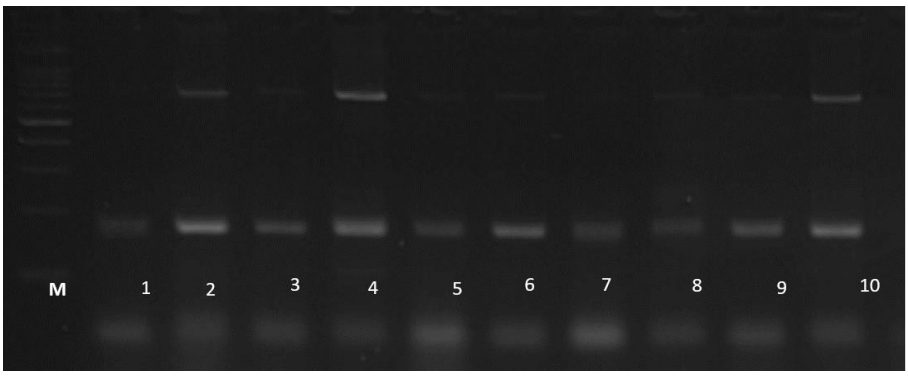

Fig 2 a: Results of SSR-PCR amplification bands of DNA extracted from new flush using SSR primer Mng SSR-14.
M: 50 bp ladder; 1: Baneshan; 2: Dilpasand; 3: Himayath; 4: Kaju; 5: Mahamooda Vikarabad; 6: Nazeem Pasand; 7: Neeleshan; 8: Neelum; 9: Pulihora; 10: Zardalu.

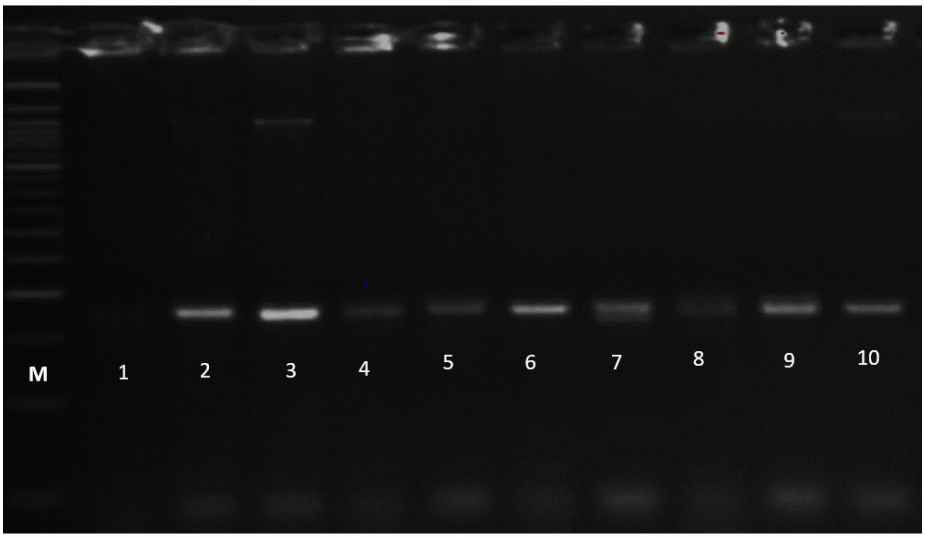

Fig 2b: Results of SSR-PCR amplification bands of DNA extracted from old leaves using SSR primer Mng SSR-14.
M: 50 bp ladder; 1: Baneshan; 2: Dilpasand; 3: Himayath; 4: Kaju; 5: Mahamooda Vikarabad; 6: Nazeem Pasand; 7: Neeleshan; 8: Neelum; 9: Pulihora; 10: Zardalu.

Different concentrations of isolated DNA from new flush and old leaves of ten mango cultivars were pure with good quality and consistent amplification products suitable for PCR applications by using SSR primers. The results of extracted DNA from new leaves and old leaves used to PCR amplification with primer Mng SSR-14 shown in Figures 2a and 2b. The amplified DNA bands of ten varieties were very clear and visible that are produced in the gel images.

Yamanaka (2006) [20] results showed that previously extracted DNA from mango leaf by using the method (Doyle and Doyle 1990) [7]. DNA isolation by using a purification kit (Mag Extractor Genome Kit) to obtain pure and good quality DNA,

but its cost ineffective. PVP 1 and 2 % and beta-mercaptoethanol 0.2 % were added to the CTAB buffer thus, making the buffer effective in reducing the polyphenols and other contaminants from mature and old leaf and also damaged leaf tissues while storage (Doyle and Doyle 1990; Howland *et al*., 1991) [7-8] According to the findings of this present experiment, the mature leaves also can be utilized to extract the DNA, whenever the sufficient young leaves are not available. Mature leaves are known to contain different contaminates like polysaccharides, secondary metabolites and also protein molecules even though can be obtained good quality of DNA by this method of extraction. Results are in the same line as that of (Sambrook *et al.*, 1981; Chatrath *et al.*, 2013; Anuradha *et al.*, 2013) [17, 3, 2].

Conclusion

The modifications made in buffer preparation described in the present study to provide the opportunity to collect good quality and pure DNA from old leaves of mango for further PCR applications. This modified extraction method has the effective and able to extract DNA from the old leaves of mango which contain higher concentrations of polysaccharides and polyphenolic compounds when enough young leaf tissue is not always available. Extracted DNA samples of ten cultivars were used to PCR amplification by using SSR primer Mng SSR-14 showed clearly visible DNA bands in both new flush and old leaves. This study suggests that extracted DNA from the modified method of CTAB is most effective and efficient in obtaining pure and clear DNA from the new leaves compared to old leaves. Extracted DNA was pure high concentration, besides being inexpensive, easy, effective, quick and simple successfully used for PCR amplification by using SSR primers.

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