JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

MODIFIED PROTOCOL FOR EXTRACTION OF DNA FROM *ANNONA CHERIMOLA* MILL. (ANNONACEAE)

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Abstract: *Annona cherimola* Mill. belonging to the family annonaceae is a popular edible fruit widely cultivated in tropical and subtropical areas. The study aims to isolate plant genomic DNA in a high-purified form for molecular-based genetic analysis, such as PCR and sequencing. The CTAB and SDS methods are popular for DNA extraction from plants, but they have limitations for polysaccharide- or polyphenol-rich tissues. The leaves of *Annona cherimola* Mill. contain high polyphenolic contents and polysaccharides, making DNA extraction challenging through conventional methods. The minor modifications made to the standard Dellaporta et al. (1983) CTAB method, including adjustments in the concentrations of CTAB, Tris, EDTA, and NaCl, proved effective in obtaining DNA of sufficient quality and quantity for molecular biology applications. The extracted DNA was suitable for PCR amplification of the maturase K (*mat*K) gene, which is crucial for genetic analysis and phylogenetic studies. The modified protocol yielded approximately 120 ng/µl of DNA, which facilitated successful gene-based PCR amplification. The findings demonstrate that the modified CTAB method is a reliable and cost-effective approach for extracting high-quality genomic DNA from *Annona cherimola* Mill., enabling further genetic studies and conservation efforts for this economically important species.

Key words: *Annona cherimola* Mill., Annonaceae, DNA extraction, CTAB,

Introduction

Isolation of plant genomic DNA in a relatively high-purified form is very essential in molecular-based genetic analysis, i.e., PCR, sequencing, etc. (Kumar *et al*., 2012). The CTAB method (Saghai-Maroof, M. A., *et. al.*, 1984) and SDS (Dellaporta, S. L., *et. al.*, 1983) are the most popular procedures for DNA extraction from plants. These methods, however, have limitations for DNA isolation from plant species that have polysaccharide- or polyphenol-rich tissues (Tanaka, J., and Ikeda, S., 2002). A number of modifications of the original protocol have been testified for the extraction of DNA from plants with high polysaccharides and polyphenol compounds (Porebski, S. *et. al.*, 1997; Michiels, A. *et. al.*, 2003; Haque, I. *et. al.*, 2008; Shepherd, L. D., and McLay, T. G., 2011).

Annonaceae family belongs to the order Magnoliales. The family Annonaceae consists of 2,500 species and 130 genera, which makes it the largest family of Magnoliales (Pirie *et al.*, 2005). After eudicots and monocots, Magnoliidae are the most diverse clade of angiosperms having 9000–10,000 species in four orders (Canellales, Piperales, Laurales and Magnoliales) (Massoni, J *et. al.*, 2014). Despite this diversity and economic importance (e.g., soursop, black pepper, cinnamon, avocado) only few reports regarding with the genomic study have been published to date (Chen, J., *et. al.*, 2019 and Chaw, S. M., *et. al.*, 2019). Investigation of such genomic data was expected to resolve the still unclear relationships of magnoliids with the respite of angiosperms (Soltis, D. E. and Soltis, P. S., 2019).

Annona cherimola Mill. (Cherimoya) commonly known as "hanumanfal" is a popular edible fruit widely cultivated in tropical and subtropical areas (Pinto, A. D. Q *et. al.*, 2005). *A. cherimola* belongs to the *Annona* genus in the *Annonaceae* family, which is the biggest family in the *Magnoliales* order (APG III, 2009). Besides its consumption as a fruit, cherimoya has long been used as traditional medicine. It provides several health benefits such as digestion, preventing high blood pressure and supporting immunity (Quilez, A. M., *et. al.*, 2018 and Albuquerque, T. G., *et. al.*, 2016). There are reports on identification, conservation and propagation of *Annona cherimola Mill.* however, there is no systematic analysis of genetic diversity in this species. In the studies of molecular biology, extraction of a good quality DNA is an important condition. The *Annona cherimola Mill.* leaves were found to contain high polyphenolic contents and polysaccharides which made the DNA extraction challenging through conventional methods. The current investigation was therefore, carried out to test the suitability of existing methods for DNA isolation and makes modifications, wherever required, to get good quality and quantity of genomic DNA.

Materials and method:

Plant material

The plant material of *Annona cherimola* Mill for DNA extraction was collected from identified cultivated trees at Narnala fort, Akot, Maharashtra, India. Total DNA was extracted from fresh leaves of each species using Dellaporta *et. al.*, method (1983) with minor modifications. Young and fresh leaves free from damage and infection was selected for isolation.

DNA isolation protocol

The leaf sample was washed with tap water, rinsed with 70% ethanol, washed with 2 to 3 times by double distilled water and dried. Midrib and petiole were removed from the leaf material and finely cut into small pieces. 150 -200 mg leaf sample was weighed and crushed in liquid nitrogen using mortar and pestle. A sufficient amount of liquid nitrogen was added whenever required for complete homogenization of leaf into a fine powder. The homogenized powder was then transferred to a 2 ml micro-centrifuge tube. Subsequently pre-warmed 800 µl DNA extraction buffer (60^{o}C, 2% CTAB w/v, 1 M Tris-base pH-8.5, 0.5 M EDTA pH-8.0 and 1.4 M NaCl and 10 µl of β- mercaptoethanol) was added to sample.

The sample was then subjected to incubation at 65^{o}C for 60 min with intermittent shaking. After incubation, the micro centrifuge tubes were allowed to cool at room temperature. 800 µl of Chloroform: Isoamyl alcohol (24:1) was added to the reaction mixture in each micro centrifuge tube. The mixture was then mixed for 10 minutes by gentle shaking. The sample was then centrifuged at 10,000 rpm at 4^{o}C for about 15 min. The supernatant obtained was then transferred to a 2 ml micro-centrifuge tube. 500 µl ice chilled Isopropanol was added to the supernatant followed by incubation on ice for 10 min. The mixture was gently shaken (not vertex) till the thread was observed and then centrifuged for 5 min at 10,000 rpm at 4^{o}C.

After centrifugation supernatant was discarded and twice the volume of ice-cold ethanol was added to it for precipitation of DNA and then centrifuged for 5 min at 10,000 rpm at 4^{o}C. After centrifugation supernatant was discarded and the pellet was then dried at 37^{o}C for 20-30 min and finally dissolved in 150 µl of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

Gel Electrophoresis

The quality of DNA was tested by Agarose (Hi-Media) Gel Electrophoresis. Samples was prepared by taking 5.0 µl of DNA and 1.0 µl of bromophenol blue dye on a ParafilmTM strip and mixed well with the help of a 10 µl volume micropipette. Samples were electrophoresed in 1X-TAE (Tris-acetate-EDTA) buffer for 20 to 25 min. at 150 V on a 0.8 % agarose gel matrix containing ethidium bromide. Gels were photographed under UV light using a Gel Documentation System (GeNei image system). All steps were repeated thrice to confirm the results.

DNA quantification:

Spectrophotometer measurements were made on the extracted DNA samples (Shamidzu) at wavelengths of 260 and 280 nm. Genomic DNA was quantified using 20 µl of sample genomic DNA diluted in 980 µl of appropriately mixed TE buffer. The quality of the DNA was assessed using the OD measurements taken at 260 and 280 nm. The DNA sample with an A260/A280 ratio of roughly 1.8 was chosen for further PCR-based investigation.

PCR analysis

The extracted genomic DNA from *A. cherimola,* was used for PCR amplification of maturase K (*mat*K) gene. The amplification reactions were done in a total volume of 50 µL consisting of 1 µl genomic DNA, matK forward primer and matK reverse Primer (Table No. 1) 2 µl each, dNTPs (2.5mM each) 1 µl, 10X TaqDNA polymerase Assay Buffer 10 µl, Taq DNA Polymerase Enzyme(3U/ml) 1 µl, molecular grade sterile water 30 ul. PCR amplifications were performed on an Eppendoff thermocycler with the following amplification conditions. 1 cycle at 94°C for 3 min, 35 cycles at 94°C for 1min, 50°C for 1min, and 72°C for 2min, followed by 1 cycle at 72°C for 7min. The amplified DNA fragments were subjected to 1% agarose gel electrophoresis (150V; 60 min) with 1X-TAE (Tris-acetate-EDTA) buffer, and visualized under UV light using a Gel Documentation System (GeNei image system). The size of the amplified DNA fragments was estimated based on 500bp DNA ladder.

Table No. 1: Primer used for PCR amplification.

No	Oligo Name	Sequence (5' to 3')	Tm (°C)	GC-Content
1	matK-F	CCTATCCATCTGGAAATCTTAG	51	40.91
2	matK-R	GTTCTAGCACAAGAAAGTCG	53	45

Result and discussion

DNA isolation

Extraction of genomic DNA, ideal for a many molecular biology applications like marker assisted analysis, phylogenetic understanding etc. is important requirement. Many commonly used protocols were tested for yield, quality and suitability of DNA for PCR amplification.

Different isolation protocols significantly influenced the quality of DNA and its gene-based amplification efficiency. The CTAB based Dellaporta *et. al.*, (1983) was most suitable for extraction of desired quantity and quality of genomic DNA from the leaf tissue (Figure 1). Approximately 120 ng/µl of DNA was obtained with this protocol, to be used for primer screening and carrying out PCR reactions, the results correlated with Nagori, R. *et.al.*, (2014). The principal modification in this method included in the extratraction buffer preparation include 1M tris instead of 100mM with pH 8.5 instead of 8, 0.5M EDTA instead of 50mM, 1.4 M Nacl instead of 500mM which resulted in formation of diatomite bridges with DNA and allowed maximum yield. Another modification involved elution of DNA with 150 µl of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). This increased the yield and quality of DNA because pooling of supernatant with more TE buffer allowed binding of more DNA with buffer. Thus, the method proved to be useful because easy to perform and inexpensive reagents, besides achieving intact high molecular weight and purity of genomic DNA. The extracted DNA proved amenable to gene-based PCR amplification. DNA extraction and purification are significant steps for molecular biology studies. The quality and integrity of the extracted DNA directly affect the results of all subsequent scientific research (Ginwal, H. S., and Maurya, S. S.,2010). In case of *Annona* species, the extraction of genomic DNA and successive amplification were difficult due to abundance of polyphenols, polysaccharides and other secondary compound. In a number of significant plant species, DNA preparations tend to be brown colored due to the oxidation of polyphenols to quinone compounds. These are powerful oxidizing agents that affect quality of DNA and protein (Loomis, W. D., 1974).

In the DNA extraction protocols use CTAB to avoid co-purifying polysaccharides from plant tissues prove to be significant (Sarwat, M. *et. al.*, 2006). As *A. cherimola* species have high amount of polysaccharides, polyphenols standard CTAB method (Dellaporta S, *et. al.*, 1983). The minor modifications in the method included use of higher CTAB concentration (2 % instead of 1.0 %), precipitation of DNA with NaCl (1.4M) and 0.5M EDTA was significant (Couch, J. A., and Fritz, P. J., 1990). The addition of NaCl at concentration higher than 0.5 M together with CTAB is known to eliminate polysaccharides (Murray, M. G., and Thompson, W.,1980).

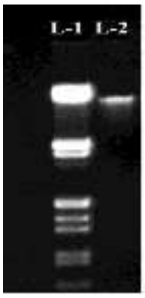

Figure No. 1: Gel images of total genomic DNA using Dellaporta *et. al.*, (1983)

PCR amplification

PCR based amplification of conserved regions (maturase K) is principally required to establish DNA barcodes for species identification, and to understand phylogenetic relationship with the other species. With universal primer *mat*k, shows good PCR amplification in *A. Cherimola* (Figure 2). The results validate the previous findings of using plastid regions (*mat*K) in angiosperms. Sequence homology of the amplified sequences was detected using Basic Local Alignment Tool (BLAST). The sequence length of *mat*k was found to be 828 bp. With *mat*k, the sequence homology of *Annona cherimola* was found to be 99. 63%. In PCR based amplification, quality of DNA plays significant role (Khanuja, S. P., *et. al.,* 1999). The sequence dissimilarity from reference sequence and phylogenetic reconstruction is the basic principle for species identification in plants (Murray, M. G., and Thompson, W., 1980).

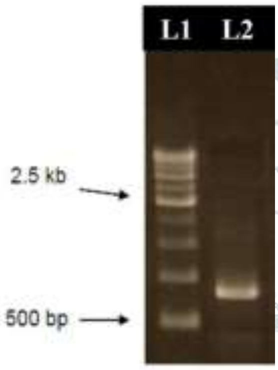

Figure No. 2: PCR amplification using *mat*k gene in *Annona cherimola* Mill.

Conclusion

This study successfully addressed the challenges of isolating high-quality genomic DNA from *Annona cherimola* Mill., a species rich in polysaccharides and polyphenols. The modifications made to the standard Dellaporta *et al.* (1983) CTAB method, including adjustments in the concentrations of CTAB, Tris, EDTA, and NaCl, proved effective in obtaining DNA of sufficient quality and quantity for molecular biology applications. The extracted DNA was suitable for PCR amplification of the *maturase K* (*matK*) gene, which is crucial for genetic analysis and phylogenetic studies. The modified protocol yielded approximately 120 ng/µl of DNA, which facilitated successful gene-based PCR amplification. The findings demonstrate that the modified CTAB method is a reliable and cost-effective approach for extracting high-quality genomic DNA from *Annona cherimola* Mill., enabling further genetic studies and conservation efforts for this economically important species.

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