

PURIFICATION OF QUALITY DNA FROM CITRUS PLANT USING IRON OXIDE NANOPARTICLE AS SOLID BASED SUPPORT

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Abstract: Purification of quality DNA is one of the essential factors affecting the success of molecular genomic studies. No single existing nucleic acid extraction method is sufficient for quality yield of DNA from high polyphenol contaminated plant materials. Here, in this study we have synchronized CTAB based lysis method with magnet mediated DNA separation utilizing iron oxide nanoparticles. An inexpensive, rapid and simple protocol has been described for extracting high quality genomic DNA from citrus leaves. Purity of the extracted DNA was revealed by the ratios of absorbance at 260/280 nm to be close to 1.80. Isolated plant genomic DNA was directly analyzed for PCR amplification which indicate freedom from common contaminating compounds. Possibly, this description is reported for the first time for the isolation of DNA from mature citrus leaf employing unmodified iron oxide nanoparticle.

Keywords: Plant, DNA isolation, Magnetic Nanoparticle, Iron oxide, PCR

INTRODUCTION

Purification of plant genomic DNA is an important requirement for genomic characterization, gene mapping, cloning and for genetic engineering. A good extraction method should yield intact and pure DNA. Different protocols for DNA isolation avoiding inhibitory compounds which compromise further analysis have been reported (Louws *et al.*, 1999). Presences of polysaccharides, phenolic compounds, secondary metabolites (Chiong *et al.*, 2017), tannins etc have been found to interfere with PCR accuracy, restriction digestion and transformation efficiency, bacterial artificial chromosome library construction (Paul *et al.*, 2014), marker-assisted polymorphism detection (Veldboom and Lee, 1994), next-generation sequencing (Collard and Mackill, 2008), southern blot hybridization (Porebski *et al.*, 1997) etc.

DNA extraction protocol generally includes two parts: Lysis of plant cell either by physical or chemical means or by enzymatic processes or in combination of three and separation of intact DNA from mixture containing lysed cells and inhibitory contaminants.

The most reliable lysis buffer for plant DNA isolation almost essentially contain CTAB as one of the major constituent, which due to cationic detergent nature precipitates DNA in low ionic concentration, but solubilise DNA in presence of higher ionic strength by chelation of acidic polysaccharides and proteins (Varma *et al.*, 2007) depending on the tissue type. Sodium chloride is also an integral part of the lysis buffer, depending on diverse polysaccharide content (Elphinstone *et al.*, 2003). Almost all methods essentially use β -mercaptoethanol during lysis due to its unique ability in prevention of polymerization. However, presence

of tannins may interfere with its activity. Guanidinium thiocyanate, as an alternative to CTAB in lysis buffer (due to its ability to bind and purify DNA molecules efficiently) also has been reported (Wang *et al.*, 2011). Different incubation temperatures were also standardized during lysis methods ranging from 4 to 96°C (Hazarika and Singh, 2018, Baranwal *et al.*, 2003). Detergents like SDS, Triton X100 (Fulton *et al.*, 1995) have also been reported along with glycerol (Hazarika and Singh, 2018) for lysis of plant samples. For specific partitioning of nucleic acids ammonium acetate (Nunes *et al.*, 2011), ice-cold isopropanol (Borah *et al.*, 2008), guanidine hydrochloride with 75% (v/v) ethanol (FU *et al.*, 2017), guanidinium thiocyanate (Rohland *et al.*, 2010), potassium acetate (Ivanova *et al.*, 2008), have been used. Precipitation of purified DNA molecules is generally obtained by washing pellets with ice chilled alcohol (100-70%). For increasing purity of the isolated DNA, further treatment with ammonium acetate, CsCl, sodium acetate, NaCl, NaOAc, GuSCN, LiCl, also have been demonstrated. Almost all methods need 3-12 hr timing and several centrifugation steps, followed by washing and drying along with proteinase K and RNase treatments are essential in case of the conventional methods.

In the last decade use of magnetic nanomaterials for DNA isolation from plant materials have also been attempted. For increasing affinity towards DNA, magnetic particles were surface functionalized with silica (Shi *et al.*, 2009), chitosan (Jiang *et al.*, 2012), carboxyphenylboronic acid (Sun *et al.*, 2015) and poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) - P(HEMA-co-GMA) (Trojáněk *et al.*, 2018). All of these protocols use a NaCl-PEG6000 solution for nonselective attachment of DNA onto

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the magnetic carrier. But none have attempted to use bare nanoparticles in this respect.

Here in we report for the first time a cost effective, environment friendly, rapid and reproducible protocol for purification of PCR ready quality DNA from citrus plant using bare iron oxide nanoparticles synthesized by a simple chemical reduction process.

MATERIAL AND METHOD

Chemicals: Ferrous sulfate (FeSO_4), potassium nitrate (KNO_3), ammonia solution (NH_4OH), Tris HCl, ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl), PEG 6000, agarose, beta-marcaptoethanol, 2-propanol, glacial acetic acid, polyvinyl pyrrolidone (PVP) and hexadecyltrimethylammonium bromide (CTAB) was purchased from Merck, India. All used chemicals

were of analytical grade. For PCR reactions dNTPs, Taq DNA polymerase, Taq buffer were obtained from KAPA Biosystems. Primers were synthesized by Eurofins India. DNA markers (Lambda DNA EcoRI/HindIII digest and BioLitProxiO 1kb) were procured from Sisco Research Laboratories Pvt. Ltd. Used Cloning vector pTZ57R/T (InsTAclone PCR Cloning Kit) was from Thermo Scientific. Used water was of MiliQ grade and for PCR reactions used nuclease free water was from Amresco.

Sample collection: The plant materials were collected from citrus orchards of Kalyani (22.9751°N , 88.4345°E) and Belpukur (23.4785°N , 88.4137°E), district Nadia, state West-Bengal, India. All the citrus leaf samples (Figure 1) were aseptically collected in sterilized plastic bags and were kept in freezer (-20°C) until use.

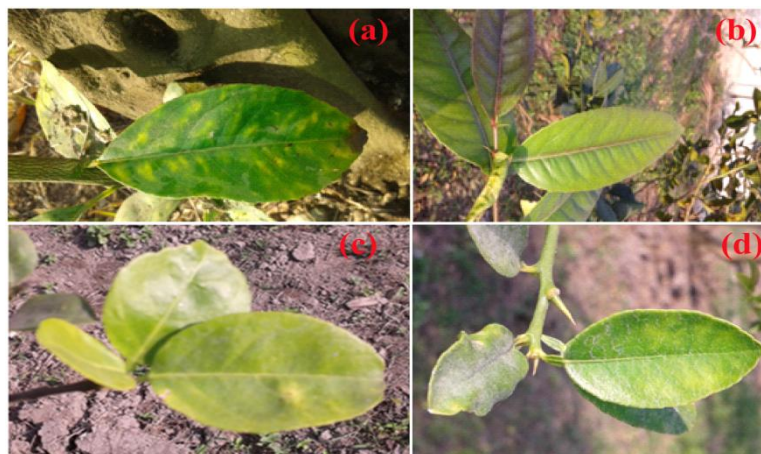


Figure 1: Citrus (Kagzi Lime) leaves from Kalyani (a, b) and Belpukur (c, d).

Preparation of iron oxide nanoparticle:

The magnetic iron oxide nanoparticle (MNP) was prepared by with some modification of previously described method (Chattopadhyay and Sarkar, 2015), reduction of ferrous sulfate and potassium nitrate mixture (1.5 gm: 1.5 gm) was done using ammonia solution (25 mL). After proper mixing for 10 min using cyclomixer thick black slurry was developed. With addition of ammonia solution to the mixture the color first turned green and finally black slurry is generated. The synthesized MNPs were kept under ammonia (highly reducing basic medium) for long storage. The MNPs were washed vigorously several times with MilliQ water prior to use. Impurities and excess ammonia was removed from the MNPs by magnetic separation. Finally, the MNPs were resuspended in 2000 μL MiliQ water. These MNPs could be stored for at least 3 months under 100% methanol (1 mL) without considerable change in morphology and magnetic properties.

Characterization of iron oxide nanoparticles: Properly diluted sample was placed on a carbon-coated copper grid (300 mesh, Applied Biosystems, India), dried in a vacuum desiccator for 72 h and morphology and size of the MNPs were analyzed by

a transmission electron microscope (TEM, Tecnai S-Twin, FEI, USA). Selected area electron diffraction (SAED) pattern of the nanoparticle was also obtained. Surface morphology of the MNPs was characterized using scanning electron microscopy (SEM, S-2300, Hitachi, Japan).

Isolation of genomic DNA from plant (citrus):

Using CTAB method – DNA was extracted from plant sample (citrus leaf) using a standard CTAB extraction method without liquid nitrogen (Murray and Thompson 1980). Purified DNA was separately dissolved in 50 μL TE buffer and finally electrophoresis was done using 0.8% agarose gel. Purification of DNA from Citrus leaf using MNP – 100 mg of finely chopped fresh citrus leaf (or stored at -20°C freezer for 2-3 months) was taken in a sterilized mortar and 2% polyvinylpyrrolidone (PVP) was added to it. 500 μL prewarmed (at 65°C) CTAB extraction buffer (containing freshly prepared 10% CTAB, 0.1 M Tris HCl of pH 8.0, 0.5 M EDTA of pH 8.0, 5 M NaCl, β -marcaptoethanol and double distilled water) was added to the chopped leaf samples to get fine paste after crushing. Again 500 μL prewarmed CTAB extraction buffer was added to the mortar and the grinded green colored solution

was resuspended in a 2 mL microcentrifuge tube. It was kept into waterbath using a flotar at 65°C for 15 min with occasional inversion of the tube in every 5 min. Then 1 mL chloroform:isoamyl alcohol mixture (24:1) was added to the samples and the tube was kept under continuous inversion condition for 15 min. The sample was centrifuged for 2 min at 5000 rpm under room temperature for precipitation of cellular debris. From the upper portion of the mixture 500 µL solution was taken carefully in a new sterilized 2 mL microcentrifuge tube and 1250 µL binding buffer (10% PEG 6000, 2.5M NaCl) was added. Now freshly prepared, properly washed, well dispersed (sonicated for 10 min at 50 MHz) 100 µL MNP (from 100 mM stock) was added to the cell lysate-binding buffer mixture and inverted for 5-10 times. The tube was incubated at room temperature for 10 min. After that using a magnetic stand (Promega Ltd.) the MNPs were immobilized and the supernatant was discarded from the microcentrifuge tube. Now the immobilized MNPs were washed twice using 95% and 70% alcohol (200 µL). Excess

alcohol was removed from the microcentrifuge tube leaving only the MNPs immobilized DNA by means of magnetic field. At room temperature the tubes were completely air-dried so that no residual alcohol was left. The bound DNA molecules were separated from the MNPs by adding 50 µL TE buffer (1 M Tris HCl, 0.5 M EDTA pH 8.0). For complete elution of DNA from MNPs, the microcentrifuge tube was incubated under continuous agitation on a waterbath (at 65°C) by tapping MNPs under an external magnetic environment. Finally, the purified DNA samples were taken in a sterilized 1.5 mL microcentrifuge tube and kept at deep freezer (-20°C) for further processing.

Amplification of extracted DNA using MNPs – DNA samples obtained from citrus leaves was used as substrate for PCR amplification.

Primer selection – PCR reaction were performed with internal transcribed spacer (ITS) region & β tubulin gene specific primer pair specific for plant (Table 1)

Table 1. Details of Primers used in the study

Target gene	Amplicon Size (bp)	Primer Name	Primer Sequence (R=A,G,Y=C/T)
ITS conserved region of plant	700	ITS1	5'TCCGTAGGTGAACCTGCGG3' (Fernández-Bodega <i>et al.</i> , 2009)
		ITS4	5'TCCTCCGCTTATTGATATGC3'
β tubulin gene of plant	1200	βtub1Planta	5'CARGGCGGCCARTGYGGBAACCA3'(Einax and Voigt, 2003)
		βtub2rPlanta	5'GGGATCCAYTCMACRAA3'

Polymerase Chain Reaction assay (PCR) – PCR amplification was carried out by standard protocol. Typically, the PCR mixture contained 5 µL DNA (~10 ng DNA) as a template, 1.0 µL of 10 mmol L⁻¹ of each primer, 2.0 µL Taq polymerase buffer, 1.0 µL of 10 mm dNTPs mix and 0.2 µL of 3U DNA Taq

polymerase. The final volume was adjusted to 20 µL by adding nuclease free water. Amplification was carried out in 35 cycles in a thermal cycler (MyiQ2, BioRad).

The following PCR conditions (Table 2) were optimized to have quality PCR products

Table 2. Details of different PCR conditions

Target Gene	Used Primer pair	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Storage
ITS region of Citrus	ITS1 & ITS4	94°C for 4 min	94°C for 1 min	56°C for 1 min	72°C for 1 min	72°C for 7 min	4°C for ∞
β tubulin of Citrus	βtub1Planta & βtub2rPlanta	94°C for 4 min	94°C for 40 sec	56°C for 40 sec	72°C for 1 min	72°C for 7 min	4°C for ∞

Analysis of PCR products – PCR products were analyzed by 0.8% agarose gel electrophoresis and stained with ethidium bromide (0.5 µgmL⁻¹) for visualization under Chemi Doc System (Bio-Rad, Munich, Germany).

Cloning of the PCR products for sequencing – The DNA fragments (PCR products) obtained from PCR using ITS specific primers (for plant) were chosen for sequencing. Appropriate bands were extracted using Himedia Gel Extraction kit. After elution the purified DNA were used as template and cloned into pTZ57R/T vector (InsTAclone PCR Cloning Kit,

Thermo Scientific). The positive plasmids were used as templates for sequencing.

RESULT

Characterization of nanoparticles – The size of magnetic nanoparticle was found to be 10 nm by transmission electron microscopy (TEM) image whereas the scanning electron microscopy (SEM) revealed spherical nature. The SAED pattern obtained during TEM analysis the nanoparticle also revealed its crystalline nature which was further confirmed from the SEM data. (Figure 2)

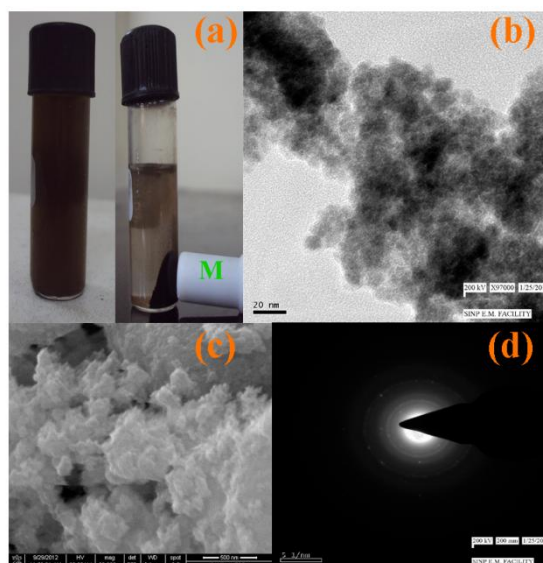


Figure 2: Characterization of MNP (a) suspension in water and immobilized in presence of magnet. (b) TEM image (c) SEM image and (d) SAED pattern.

DNA isolation study – Here we compared the qualitative and quantitative nature of CTAB based protocol for DNA isolation with the magnet mediated separation procedure using MNPs from plant (citrus) samples (Table 3). The electrophoretic study (Figure 3) revealed high molecular weight DNA content around 21 kbp, resembling plant genomic DNA. In case of magnetic separation, no RNA contamination

was present whereas a noticeable RNA smear was observed in case of conventional CTAB based DNA isolation process. Presence of protein contaminant in the purified DNA samples was evaluated by A260/A280 ratio, and in case of MNP mediated separation it was found to be 1.75 compared to 1.55 for conventional method.

Table 3. Concentration and purity of Extracted DNA

Method for DNA isolation	Sample source	Sample ID	Sample weight (mg)	Time for DNA isolation	Sample quantity μL	Concentration of obtained DNA ($\text{ng}\mu\text{L}^{-1}$)	Purity of extracted DNA (A_{260}/A_{280})
Magnetic bioseparation	Citrus Plant Leaf	CP1	100	45 min	50	253	1.75
	Citrus Plant Leaf	CP2	100	45 min	50	235	1.72
	Citrus Plant Leaf	CP3	100	45 min	50	245	1.78
Conventional method	Citrus Leaf	CCP	100	24 hr	50	200	1.55

Amplification of isolated DNA – For quality analysis, MNPs mediated extracted DNA samples were subjected to PCR amplification. Agarose gel image (Figure 3) of PCR products extracted by MNPs method reveals that DNA were successfully amplified using ITS and Tubulin specific primers. For investigation of further compatibility towards other molecular techniques, at least one positive clone from amplified DNA sample was sequenced.

The sequences were aligned using the CLUSTAL W program with the deposited sequences from the database of the National Center for Biotechnology Information using a BLAST search and deposited in NCBI Genbank. Analysis of the sequences with already available submissions revealed 99% similarity (Table – 4), suggesting the nanoparticle mediated plant DNA isolation method to be a successful one.

Table 4. Details of sequenced DNA samples

Organism	Target Gene	Amplicon Size (bp)	Genbank Accession Number
<i>Citrus aurantifolia</i> (Kagzi Lime)	ITS conserved region	700	MF797955, MF797954, MF797953
<i>Citrus aurantifolia</i> (Kagzi Lime)	β tubulin gene conserved region	1200	to be submitted

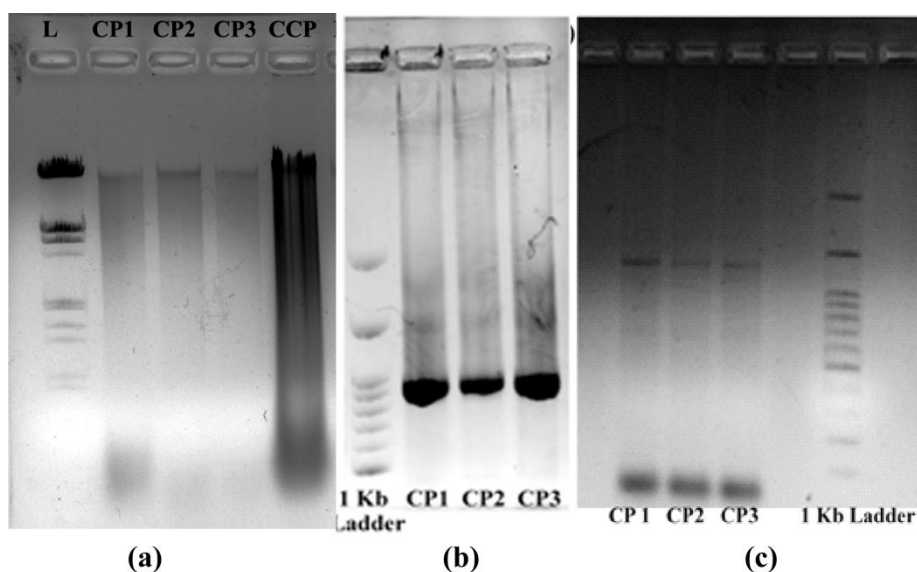


Figure 3: Agarose gel electrophoresis image of genomic DNA:

DNA molecular weight marker (λ phage DNA/*Hind*III digest) (L), DNA isolated using MNPs (CP1, CP2, CP3), conventional CTAB method (CCP) from citrus leaves.

PCR amplicons using ITS and (c) Tubulin specific primers (CP1, CP2, CP3) with 1 kbp DNA ladder.

DISCUSSION

Various methods are available for isolation of DNA from different plant samples but purification of quality DNA from plant materials is very difficult, due to presence of polyphenols. Most of the methods used for extraction of plant DNA could not satisfy for molecular analysis due to variable efficiencies. Quality and quantity of isolated DNA is affected by many parameters like proper cell lysis, absorption of DNA to a particular matrix, presence of impurities causing damage of DNA etc. DNA extraction protocol has two common steps: lysis and purification of inhibitor free high molecular weight DNA for subsequent molecular analysis. Thus, to get quality DNA, it is important to select particular steps with cost effectiveness and with minimum time. Our present study has compared the efficiency of conventional (CTAB) plant DNA isolation protocol with MNPs mediated modified CTAB method in terms of rapid, cost effective and purity of extracted DNA for molecular analysis.

MNPs mediated protocol showed some advantages over the conventional CTAB method. It is cost effective and less time consuming. The comparison of both methods is tabulated in terms of quality, quantity and protocol time (Table 3), which indicates that multi-stepping and costly conventional procedure can be avoided by our MNPs mediated plant DNA isolation protocol. Due to presence of surface charge and large surface/volume ratio, iron oxide nanoparticles are highly interactive towards DNA molecules by electrostatic interaction (Ito *et al.*, 2005, Paul *et al.*, 2014). Our as synthesized iron oxide nanoparticles of 10 nm size showed magnetic properties, which is important for DNA separation

amongst other impurities (Bandyopadhyay *et al.*, 2011).

Extraction of DNA from plant materials is difficult due to interference of various polyphenols. To remove these inhibitors, a number of plant DNA extraction methods are already described which include enzyme treatments, bead beating, freeze thawing along with the use of liquid nitrogen, make these methods costly.

Our MNP preparation is easy and required only common laboratory chemicals (iron sulfate, potassium nitrate, ammonia solution) and common instruments (waterbath, cyclomixer). In our protocol, the grinding process for plant cell wall rupturing does not require liquid nitrogen, which is an integral part of almost all protocols involving CTAB. In the conventional process use of high speed centrifuge (8000-14000 rpm) is essential in many steps, whereas in our protocol involvement of centrifugation step is only once (during precipitation of cellular debris). This step of centrifugation can be carried out even in 5000 rpm (which can be achieved in any table top centrifuge). Only a simple magnet is being used for capturing and immobilization of magnetic nanoparticles bound DNA. The total procedure can be managed in a single centrifuge tube making it convenient for preparation of large number of samples easily. Due to surface charges, the bare iron oxide nanoparticles were specifically bound towards free DNA by electrostatic attraction. Thus, no other impurities (RNA, Protein) were appeared in the electrophoretic study. However, during conventional processes the DNA is precipitated by the action of isopropanol, needed at least 4-12 hr, whereas in our process the DNA can be precipitated down within 10 min using MNP at room temperature. Purity of the isolated DNAs was

estimated both by nanodrop and through electrophoresis, in both cases the average A260/280 was 1.75 with overall yield of 1.2-1.5 microgram DNA. As proof of quality checking, the extracted DNA by MNP method was subjected to PCR amplification. For proper amplification, inhibitor free quality DNA extraction has utmost importance. Our method successfully overcome such limitations and gives a good amplified product. Thus, MNP mediated protocol proved to be unique which can be extended further to carry out molecular biological applications (e.g detection of plant diseases) in a rapid manner.

CONCLUSION

A cost effective, robust and rapid method for isolation of genomic DNA from plant (Citrus) has been described. This protocol not only combines the traditional CTAB based method with some modifications but also uses nanoparticle as an integral part for isolation of DNA. Good quality and highly concentrated genomic DNA samples were obtained by use of this facile method. As this process is beneficial with the potential to be used for isolation of genomic DNA from plant samples using simple instruments, so this protocol is applicable for large scale DNA isolation processes even in remote areas where sophisticated laboratory facilities are unavailable.

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