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Isolation of genomic DNA from groundnut plant by modified, rapid and efficient protocol

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Abstract
DNA isolation is prerequisite to study the crop at molecular level. However, DNA (Deoxyribonucleic Acid) isolation is difficult in groundnut due to presence of large amount of polyphenols and polysaccharides. The age and growth stage of the plant also affect the DNA purity during isolation. In this study, newly expanded leaves and stems of five days old seedlings and matured leaves of 20 days old seedlings were used for optimization of extraction protocol. High quality DNA was obtained from all plant materials by this modified CTAB (Cetyltrimethylammonium bromide) protocol without separate purification. Optimized protocol was used to isolate DNA from newly expanded leaves of 5 days old seedlings of 100 groundnut genotypes. DNA obtained ranged from 6.3-22.1 µg/ g of sample when quantified by Nano Drop spectrophotometer and was able to amplify with Simple sequence repeats (SSR) primers during Polymerase Chain Reaction (PCR).

Keywords: Groundnut, DNA extraction, CTAB, polyphenols

Introduction
Groundnut is an important oil seed crop grown in Asia and Africa. It is mainly grown for edible oil extraction and also used for table purpose and confectionary. Due to climate change, there is drastic increase in biotic and abiotic stresses, which affect production and quality of groundnut. To overcome these constrains apart from conventional techniques, molecular techniques are being used. DNA extraction is prerequisite work for all molecular techniques of crop improvement such as, identification of transgenic plants (Keshavareddy *et al*., 2013) [4], mapping (Varshney *et al*., 2009) [15], cloning (Li *et al*., 2016) [6], identification of resistant genotypes for biotic and abiotic stress (Yol *et al*., 2016) [16], etc. In plants, a breakthrough in DNA extraction came in 1980 with the development of the CTAB protocol (Murray and Thompson, 1980) [9]. Over the years, since publication of the original protocol, various modifications have been designed according to the crops involved and to reduce time and cost of routine DNA extraction (Allen *et al*., 2006) [1]. Consistent isolation of best quality DNA from groundnut is problematic, particularly due to the presence of polyphenols and polysaccharides. Inconsistencies in extraction results can be attributed to the age and growth stages of the plant material analyzed. Mature leaves have higher quantities of polyphenols, tannins, and polysaccharides that can contaminate DNA during isolation (Roomi *et al*., 2013) [11]. The presence of polyphenols, which are powerful oxidising agents present in many plant species, can reduce the yield and purity of extracted DNA (Porebski *et al*., 1997) [10]. Various modifications have been made to isolate DNA from plants containing high polyphenols, polysaccharides and essential oils (Lodhi *et al*., 1994; Khanuja *et al*., 1999; Sharma *et al*., 2002; Cheng *et al*., 2003; Deshmukh *et al*., 2007; Sahu *et al*., 2012; Turaki *et al*., 2017) [7, 5, 13, 2, 3, 12, 14]. The biochemical composition in plant tissues of different species is expected to vary considerably and hence may not yield optimal DNA from one isolation protocol (Khanuja *et al*., 1999) [5]. There are available protocols for DNA isolation in groundnut but they require isolation of crude DNA first and then its purification, which is time consuming. Designing of rapid and efficient protocol is a necessity if large number of samples is involved, like in mapping; diversity analysis, and Marker Assisted Selection program (Mace *et al*., 2003) [8]. The modified procedure here does not involve a separate purification procedure hence, is rapid and inexpensive and produces quality DNA in sufficient quantity that is suitable for PCR.

Materials and methods
Chemicals, Consumables and Equippments
Disodium ethylenediamine (EDTA), Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), Sodium Chloride (NaCl), Sodium hydroxide pellete, Hydrochloric acid, Cetyltrimethyl ammonium Bromide (CTAB), β-mercaptoethanol, Polyvinylpyrrolidone (PVP), Chloroform,

Isoamyl alcohol, Phenol:Chloroform:Iso-amyl alcohol (P:C:I) (25:24:1), Isopropanol, Ethanol, Liquid Nitrogen, RNAase, Nuclease free 2ml Polypropylene snap-cap micro centrifuge tubes, Nuclease free wide bore micropipette tips to fit required micropipette, Hand gloves, Pestle and Mortar, Spatula, Water bath, Microcentrifuge capable of spinning 2ml tube at 12000rpm, Micropipettes, Gel Doc (Gel Documentation System)

Plant materials

100 groundnut genotypes. Two genotypes were used for optimization of protocols.

i) Newly expanded leaves of five days old seedlings after emergence, ii) Stems of five days old seedlings and iii) Matured leaves of 10 days old seedlings.

Protocols

- DNA extraction method was modified from Lodhi *et al*., 1994 [7] for the extraction of total genomic DNA from Groundnut plant sample. Same extraction procedure was followed for each plant materials.
- Prepare fresh extraction buffer (2% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl and 2 µl/ml β-mercaptoethanol). Add β-mercaptoethanol newly before use.
- Add 3% (w/v) PVP to the extraction buffer as it binds with polyphenols and hence removes it during solvent extraction.
- Grind 0.2 g of plant sample in liquid nitrogen using a pre-chilled pastel and mortar.
- Transfer[1] the fine powder to 2 ml polypropylene centrifuge tube containing pre warmed 700µl CTAB DNA extraction buffer. Mix the powder thoroughly by gently inverting the tubes.
- Incubate the tubes at 65 °C for 45 minutes in thermostatic water bath for cell lysis. Mix the content in tubes after every 15 minutes by inversion during incubation.
- After incubation, allow the tubes to cool at room temperature.
- Add 700µl P:C:I 25:24:1 (v/v/v) into the incubated extract and mix gently by at least 20-25 inversion.
- Centrifuge the tubes at 12000rpm for 10 min at room temperature in high-speed centrifuge for first clarification.
- Carefully transfer the upper aqueous phase[2] in new 1.5ml tubes with the help of pipette.
- Add equal volume (700µl) of chloroform:isoamylalcohol (C:I) 24:1 (v/v) to the recovered aqueous phase and mix gently by inversion and centrifuge at 12000rpm for 10 min at room temperature in high speed centrifuge for second clarification.
  (If the recovered aqueous phase is translucent then repeat the clarification procedure till the recovery of transparent aqueous phase)
- Now carefully transfer upper transparent aqueous layer into a fresh micro centrifuge tube and then add 150µl of 5M NaCl and 450µl of cold isopropanol (-20 °C).
- Mix the mixture well by gently inverting the tubes 3-4 times[3] and incubate at -20 °C for one hour.
- Centrifuge the tube containing mixture at 10000rpm for 10 min at 4 °C in refrigerated centrifuge to pellet the nucleic acids.
- Decant the supernatant carefully and pour 100µl of ice-cold 70% ethanol to the tubes to wash the pellet.
- Centrifuge the tube at 10000rpm for 5 min and then decant ethanol carefully. Wash the pellet one more time with 70% ethanol in same manner.
- Air dry the pellet[4], then re-suspend in 100µl 1x TE buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA) or distilled water[5] and stored at -20 °C until use.
- Add 5µl of RNAase (10mg/ml) in each tube containing isolated re-suspended DNA and incubate at 37 °C for 30 min.
- Treated DNA was then quantified using a NanoDrop 1000 spectrophotometer (Marshall Scientific). DNA purity is the ratio of spectrophotometric absorbance of DNA at λ=260/280 nm and was checked on 0.8% (w/v) agarose gel.
- Extracted DNA was diluted appropriately in nuclease-free sterile distilled water before using for PCR.

Notes

1. Transfer the powdered sample immediately else it will absorb atmospheric moisture.
2. While transferring the supernatant, do not disturb the interface and do not pipette even fraction of debris.
3. At this point DNA will appear as white thread like structure, so do not shake vigorously as DNA is vulnerable to be sheared.
4. Pellet should not be over dried as it will be difficult to dissolve it. At the same time it should not be under dried either as it will have ethanol content.
5. If DNA is for immediate use, it is better to dissolve in sterile, nuclease free water and if DNA is for storage for longer period then it must be dissolved in TE buffer. DNA re-suspended in TE buffer should be diluted properly before downstream use like PCR as EDTA present in buffer will interfere with DNA polymerase enzyme.

Genomic DNA of 100 genotypes was isolated by using above protocol with newly expanded leaves of five days old seedlings. Quality of isolated DNA was checked by using it as a template in PCR amplification with SSR primers. Total volume of reaction mixture for DNA amplification was 20µl, containing Taq Buffer B (Genei) (10X) 2µl, MgCl2 (25mM) 2µl, dNTP mix (10mM) 1.6µl, Primer Pair (10pico mol) 1.5µl each, Taq DNA Polymerase (3U/ µl) 0.33µl, Sterilized distilled water 9.07 µl, Template DNA (50ng) 2µl (Yol *et al*., 2016) [16]. The PCR conditions set for amplification were initial denaturation at 94 °C for 3 minutes, 35 cycle of denaturation at 94 °C (45sec), annealing at 56 °C (45sec), extension at 72 °C (1min) and final extension at 72 °C for 8min. Amplified band was then separated by electrophoresis on 2.5% agarose gel dissolved in 1X TBE (Tris-Borate-EDTA) buffer, stained with 5µg/ml ethidium bromide.

Results and Discussion

DNA isolated from all plant materials, were of good quality as well as quantity. Quantity of the DNA was checked by running the sample on 0.8 % agarose gel (Figure: 1). There was no need of purification of DNA as they were amplifying well with SSR markers in PCR. Quantity of DNA was less in newly expanded leaves and stems of 5 days old seedlings compare to the mature leaves of 10 days old seedling. This finding was in accordance with Lodhi *et al*., 1994 [7]. In case of mature leaves, during DNA extraction, two clarifications with P:C:I were done followed by one C:I treatment since the upper aqueous solution was cloudy. But in case of materials of 5 days old seedlings, only one P:C:I and one C:I treatments were required.

This protocol was verified by isolating DNA from newly expanded leaves of hundred groundnut genotypes. Quantity of DNA was checked by NanoDrop 1000 spectrophotometer and it ranged from 6.3 to 22.1µg/ g of sample with absorbance ratio ranged 1.72 – 1.89(Data not included) which indicated good quality DNA and this finding was similar with Sharma *et al*., 2002 [13]. The authenticity of the DNA was checked by using the isolated DNA as template in PCR amplification using SSR primer PM36, which resulted in amplification of genomic DNA in the expected molecular weight range (Figure 2 (a,b,c,d)).

High concentration of PVP helped in removal of polyphenols from groundnut DNA which is supported by Khanuja *et al*., 1999 [5]. In this protocol P:C:I and C:I were used in clarification process instead of Chloroform:Octanol since phenol dissolves more organic matter and hence removes protein contaminants (Roomi *et al*., 2013; Turaki *et al*., 2017) [11, 14]. High concentration of salt was used to in extraction of groundnut DNA which helped in removal of polysaccharides as reported by Khanuja *et al*., 1999 [5].

This method is rapid, simple, and efficient for isolating DNA from groundnut plants rich in polyphenols and polysaccharides. It does not require separate purification of DNA which makes it less time consuming. Most of the PCR based molecular methods of crop improvement like screening of genotypes, marker assisted selection, molecular diversity analysis need handling of large population so this protocol will help the researchers to save time.

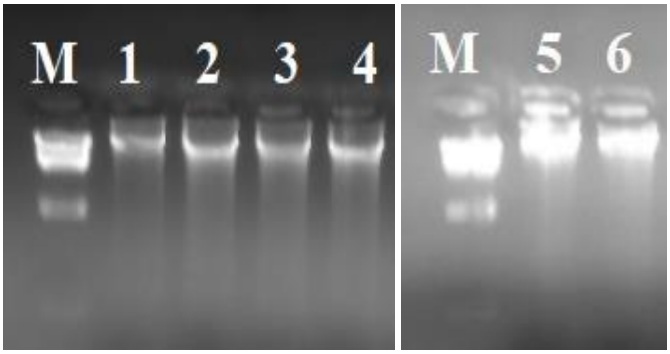
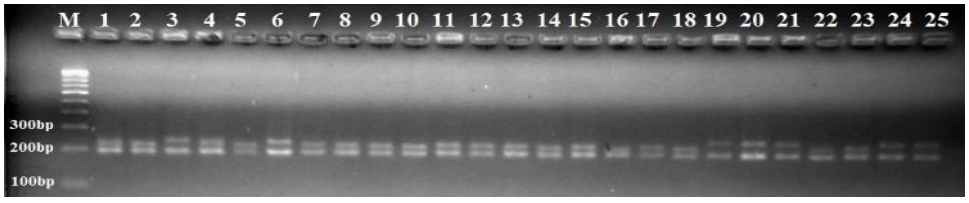
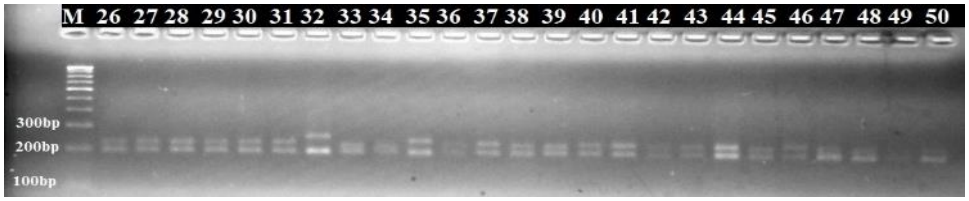


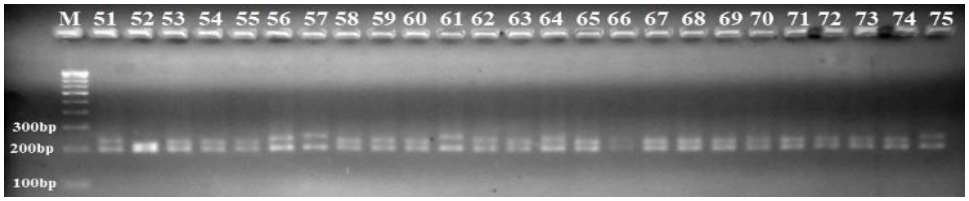
Fig 1: 0.8% Agarose gel, stained with 5µg/ml Ethidium bromide and illuminated by UV rays.
M: λ-DNA digested with Hind III; 1, 2: DNA isolated from newly expanded leaves of 5 days old seedlings; 3, 4: DNA isolated from stem of 5 days old seedlings; 5, 6: DNA isolated from matured leaves of 10 days old seedlings



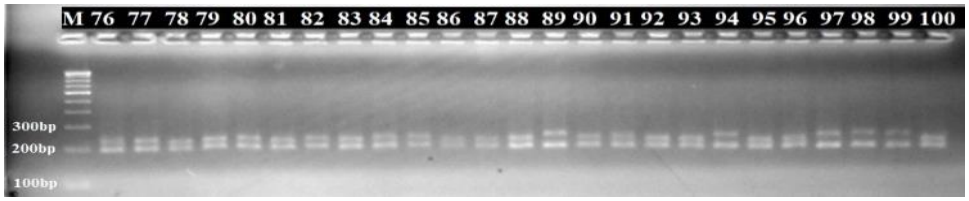
a



b



c



d

Fig 2: (a,b,c,d): 2.5% agarose gel stained with 5µg/ml Ehidium bromide and illuminated with UV rays, showing PCR-amplified products obtained from DNA isolated from newly expanded leaves tissues, using SSR marker- PM36. M- 100bp ladder, 1 to 100- Amplified PCR products of 100 groundnut genotypes.

Declaration
The authors declare no conflict of interest.

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