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COMPARATIVE STUDIES OF DIFFERENT DNA EXTRACTION METHODS FOR PCR – BASED ASSAY IN *ARACHIS HYPOGAEA* (L.)

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Abstract:

High quality DNA extractions are a prerequisite for genetic studies for a variety of plants including Arachis hypogea (L.) Nowadays, there are great number of plant DNA extraction method and commercially available extraction kit are also becoming more and more popular. It appears that different procedures work best for different plant groups. Thus in the genetic studies of A.hypogea, choosing CTAB method to choose becomes a concern. The DNA extracted by this method from (Control and Seaweed Liquid Fertilizer (SLF) treated fresh young leaf tissue of A.hypogea was analysed according to their cost and time, yield, purity, integrity and PCR (Polymerase Chain Reaction) based downstream analysis._The quality and quantity of isolated DNA was measured by Nano photometer. The absorption value A260/280 was calculated. Based on OD values the 10% and 25% of SLF treated plants showed pure and contaminant free DNAs when compared to control plants. Further the isolated genomic DNA was checked with 0.8% agarose gel electrophoresis stained with ethidium bromide to check the DNA quality. The gel was photographed under gel documentation system. In addition, the quantity and quality of the DNA extracted by this method were high enough to perform hundreds of PCR based reactions.

Key words: *DNA purification, Nano photometer, PCR, extractions.*

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INTRODUCTION:

Peanut (*Arachis hypogaea* L.) is an important crop for edible oil and protein, which is grown mainly in semi-arid tropic and sub-tropic areas of 109 countries around the world. A variety of molecular, chemical, and morphological descriptions are used to characterize the genetic diversity among and within crop species. Molecular marker techniques including random amplified polymorphic DNA (RAPD) [1] have been used to study polymorphism in groundnut. Different protocols for DNA extraction have been applied to control and SLF treated leaves which were modified to provide DNA extraction protocol, suitable for several kinds of genetic studies in plants[2], DNA extraction is an important step in molecular assay and plays a vital role in obtaining high resolution results in gel-based systems, particularly in the case of cereals with high content of interfering components in the early steps of DNA extraction. A prerequisite for taking advantage of these methods is the ability to isolate genomic DNA of superior quality and quantity for analyzing through Polymerase Chain Reaction (PCR), The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification. Certain polysaccharides are known to inhibit RAPD reactions [3].

MATERIALS AND METHODS:

Plant Material

The samples of young and tender leaves of control and SLF treated plants of Arachis hypogaea were collected from Botanical garden, Department of Botany, Annamalai University, Annamalai Nagar, Tamil Nadu. After washing the plant tissue with sterile water and subsequently with 70 per cent alcohol, 1g of fresh leaf tissue of species was taken and then it was chopped into fine pieces and subjected to genomic DNA isolation. Genomic DNA was extracted from control and SLF treated leaves by adopting the modified CTAB method outlined by [2] without using liquid nitrogen. Genomic DNA yield was expressed as ug DNA per mg of leaves tissue.

Testing DNA Isolation methods

Method 1: HipurA Plant Genomic DNA extraction Kit (Himedia, Cat#MB502-PR)

Method 2: Described by Doyle and Doyle 1987. Modified CetylTrimethyl Ammonium Bromide (CTAB) extraction protocol was used. Which was applied in many palnt species. Extraction buffer in this protocol was included: 2% CTAB, 100mM TrisHCl, 2% PVP, 1.4M NaCl, 20mM EDTA (pH: 8.0), 2% B-mercapto ethanol, chloroform : Isoamyl alcohol (24:1), Isopropanol, RNase, 70 % ethanol, TE buffer (10mM TrisHCl, 1mM EDTA pH:8.0), immediately prior to use.

Method 3: Described by modified CTAB Buffer. in this protocol was included: 50mM EDTA, 120 mM TrisHCl, 1.5M NaCl, , 1.5 b mercapto ethanol .

Modified DNA extraction method of CTAB was as follow:

(1) Preheat CTAB buffer in water bath at 65*C. Grind 1g of young leaves to fine powder in ice cold condition in the presence of 100mg PVP (Poly Vinyl Pyrrolidone) by using pre chilled mortar and pestle (-40*C/-80*C). (2) Transfer the contain in 2 ml micro centrifuge tubes and suspend in two volumes of CTAB buffer. (3) Invert and mix gently and incubate at 60*C for 30 min. (4) centrifuge the suspension at 10,000 rpm for 10 min at room temperature. (5) add 1.5 mL of extraction buffer and incubate at 60*C for 30 min (6) Centrifuge at 10,000 rpm for 10 min at room temperature. (7) carefully transfer the aqueous phase into a new tube. (8) add double volume of chloroform:Isoamyl alcohol (24:1) and invert gently 10 to 15 times and centrifuge at 10,000 rpm for 10 min. (9) Add double volume of chilled isopropanol and keep at -20*C for one hour to precipitate the DNA. (10) Centrifuge at 10,000 rpm for 10 min and discard the supernatant. (11) To the pellet, add 70% chilled ethanol and spool out the pellet carefully and centrifuge again at 10,000 rpm for 10 min. (12) Discard the supernatant and vacuum dry or air dry the pellet at room temperature. (13) Add 100uL of high salt TE buffer. (14) Add 3uL RNase and keep at 37*C for 30 min. (14) Add 3 M Sodium acetate (15) Spool out the DNA, wash in 70% ethanol, air or vacuum dry. (16) add 30 to 50 ul (depending upon the pellet) of TE buffer to dissolve the precipitate. (17) store at -20*C/-40*C till further use.

Qualitative and Quantitative analysis of Extracted DNA

The yield of DNA per gram of control and SLF treated leaves tissue extracted was measured using a Nano photometer (Implen, P360 Version 1.2.0) at 260nm. The purity of DNA was determined by calculating the ratio of absorbance at 260nm to that of 280nm. DNA concentration and purity was also determined by running the samples 0.8% agarose gel based on the intensities of band when compared with the lambda DNA marker (Used to determine the concentration).

Optimization of Random Amplified Polymorphic DNA (RAPD) Reaction

The PCR amplification reaction was carried out with ten Oligo nucleotide OPA primers from 1 to 10 series obtained from GeNei (Bangalore). Each

20 ul reaction volume containing 10mM TrisHCl (pH 8.3), 2.5mM Mgcl2, 25mM dNTPs mix, 0.2uM of each primer, 10x Taq buffer, 1U of Taq DNA Polymerase and 50 ng of template DNA. RAPD-PCR was performed in Master cycler nexus (Eppendorf) for 40 cycles consisting of denaturation at 94*C for 45 sec, annealing at 38*C for 50 sec, and extension at 72*C for 60 sec. the final extension was carried out at the same temperature for 10 min and the hold temperature of 4*C at the end. The PCR amplified product were electrophoresed on 2% (w/v) agarose gels, in 1x TAE buffer at 65 V for 3 hrs and then stained with ethidium bromide (0.5ug/ml). Gels with amplification fragments were visualized and Photographed under UV gel documentation system (Alpha Innotech). Lambda DNA was used as molecular marker (GeNei, Bangalore) to know the size of the fragments.

RESULT AND DISCUSSION

Choice of the material

RAPD – PCR and it showed that 10 primers could reveal some polymorphism in the amplified DNA sample ranged from 100 to 1000 bp in size. All the 10 peanut genotypes were examined for DNA polymorphism out of 10 primers used 8 primers produced amplification where as 2 primers OPA-9 and OPA-10 did not show any amplification out of 8 primers showed variable degree of polymorphism ranged from 25percent to 100percent single monomorphic bands were found in OPA-6 and OPA-8 in lane maximum numbers of monomorphic , polymorphic and rare bands were observed in fig 3a Similar results have already been reported by [4] that RAPD bands amplified by one primer vary in intensity from those amplified by another primer. Earlier, random amplification of polymorphic DNA analysis was conducted on the genetic resources of Plantago sp. To access genetic variability.[5]. His conclusion confirmed to a wide variation of phenotype in the cultivated peanut, which contradicted with some previous studies [6] Generally, natural plant tissue are not preferred for DNA extraction due to mainly to the presence of high concentration of polysaccharides, polyphenols and other secondary metabolites [2-3].

Table 1. Different Concentration of Modified CTAB Method

S.No	Name of the Solutions	CTAB - Standard Concentration Method	CTAB - Modified Concentration Method
1.	EDTA	20mM	50mM
	Tris –HCl	100mM	120mM
	NaCl	1.5M	1M
	B-mercapto ethanol	2 %	1.5%

Table 2.Comparison of Quality and Quantity of genomic DNA isolated from Control and SLF Treated Leaves of Arachis hypogaea.

S. No	Methods	Control leaves		SLF Treated Leaves	
		DNA yield (ug/g)	A260/A280	DNA yield (ug/g)	A260/A280
1	Commercial Kit	1.54±0.04	1.57±0.04	1.94±0.05	1.97±0.05
2	Standard CTAB	17.72±1.94	1.64±0.07	22.12±1.85	1.74±0.07
3	CTABModified Method	**62.61**±1.93	**1.84**±0.05	**88.11**±2.81	**1.92**±0.05

The results are mean of triplicates determination ± standard deviation.**Data are means** ± SD (n=3).

Absorption spectrum of DNA isolated from SLF Treated Leaves of Arachis hypogaea by using Modified CTAB Method

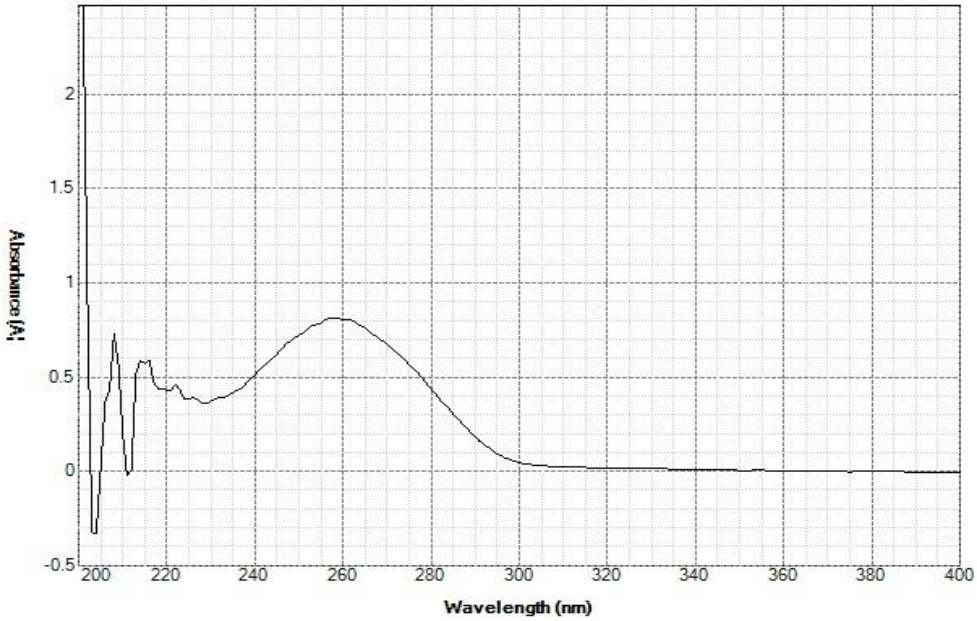
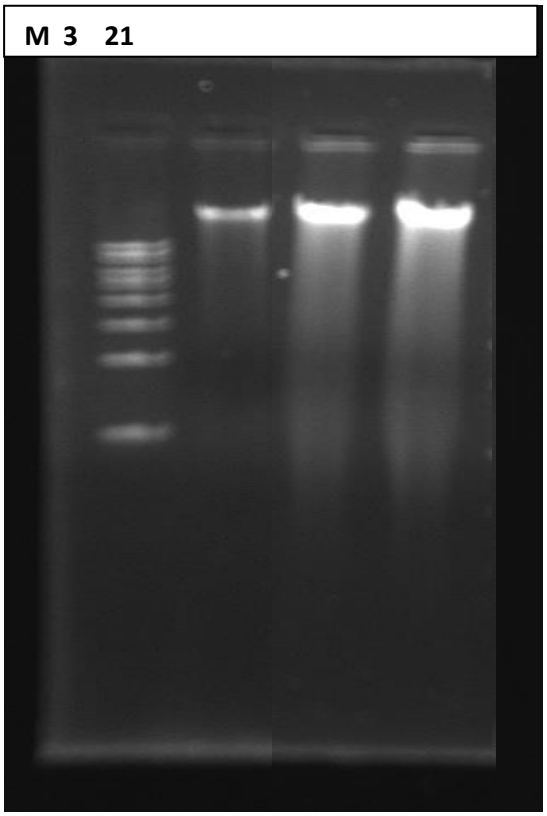


Table 3.Details of Primers using RAPD-PCR analysis on DNA from Arachis hypogaea by using CTAB Modified Method.

S.No	Primer	Sequences
1	OPA-01	5’-CAGGCCCTTC -3’
2	OPA-02	5’-TGCCGAGCTG -3’
3	OPA-03	5’-AGTCAGCCAC -3’
4	OPA-04	5’-AATCGGGCTG -3’
5	OPA-05	5’-AGGGGTCTTG -3’
6	OPA-06	5’-GGTCCCTGAC -3’
7	OPA-07	5’-GAAACGGGTG -3’
8	OPA-08	5’-GTGACGTAGG -3’
9	OPA-09	5’-GGGTAACGCC -3’
10	OPA-10	5’-GTGATCGCAG -3’

Figure 1. Genomic DNA of Arachis hypogaea resolved in 0.8% of Agarose Gel Electrophoresis



M-1Kb DNA Ladder
1. OPA-01
2. OPA-02
3. OPA-03
4. OPA-04
5. OPA-05
6 . OPA-06
7. OPA-07
8. OPA-08
9. OPA-09
10.OPA-10

GENOMIC DNA RESOLVED ON 0.8% AGAROSE

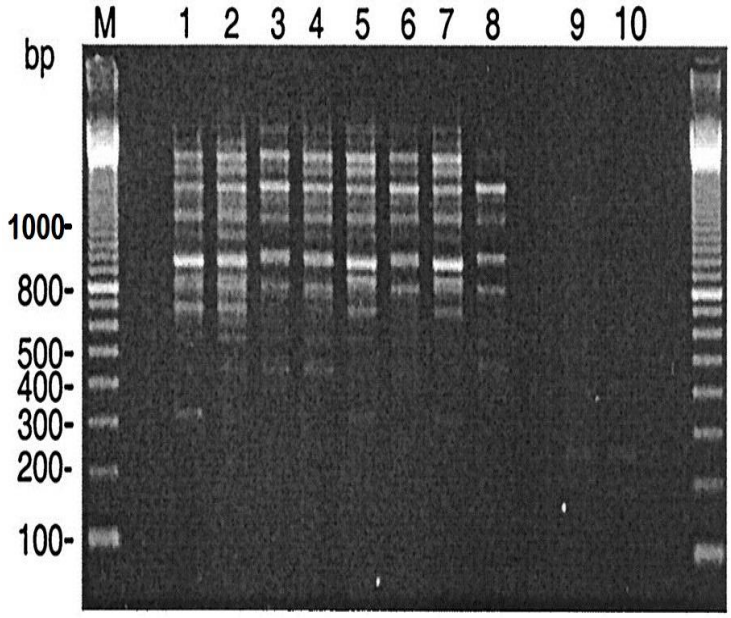
M-1Kb DNA Marker

1-HipurA Kit Method

2-Standard CTAB Method

3-Modified CTAB Method

Figure 2. Polymerase chain reaction using ten primers on DNA from leaves samples of *Arachis hypogaea* by usingCTAB Modified Method.



The assessment of the purity of nucleic acid sample in often performed by a procedure commonly referred to as the OD260/280 ratio. Although this procedure was first described by Warburg & Christian [7] as a means to measure protein purity in the presence of nucleic acid contamination, it is most commonly used today to assess purity of nucleic acid sample (Held et al 2006). A pure sample of DNA has the ratio at 1.8 (Chen et al 2010).

The mean OD260/280 ratios for three methods described by HipurA kit, CTAB and CTAB Modified method were higher than 1.9. in these three methods RNA disposal was not involved, hence there existed some RNA residues, as determined by the electrophoresis on agarose gel (Figure1, there were clear main bands observed). The mean OD260/280 ratios of CTAB method, HipurA kit method were observed the ratio between 1.4 to 1.7. it means, the extracted DNA was relatively free from RNA and Protein contamination. RNAse was used to remove RNA from DNA in all the three procedure.

The integrity i.e presence of high molecular genomic DNA, was determined by electrophoresis 0.8 % agarose gel. High molecular DNA bands with no smear were obtained from all methods while DNA isolated from the methods described by CTAB, HipurA kit method and CTAB Modified method showed high molecular DNA bands with smear with the bottom of the lane 1, 2, 3 (Figure 1), demonstrating that the DNA were intact but there existed some RNA or Protein residues.

There are at least three main contaminants associated with plant DNA: Polyphenolic compounds, polysaccharides and RNA[8] . Polysaccharides, which are difficult to separate from DNA [9] interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases[10]. More over Lodhi et al 1994 found that when polysaccharides were not removed, the DNA would not amplify in PCR reaction. The PCR reaction using ten randomized Operon Oligonucleotide RAPD primers (Table 3) was carried out to compare the DNA extracted quality (Figure2). The amplification of *Arachis hypogaea* DNA was observed only on the OPA-1 to OPA-8 series of primers. There is no amplification for OPA-9 and OPA-10th series of Primers. As the observed DNA extracted from the selected CTAB Modified method, had good amplification and also had the good banding pattern (Figure2).

There are two different view points on the effect of RNA residue. Some researchers hold the opinion that contaminants like RNA often inhibit restriction endonucleases digestion and / or PCR amplification [7,8] There is also new data indicating that RNA contamination can reduce the effectiveness of many enzymatic process ([11-12]. Further more, the RNA degrades at high temperature in the presence of magnesium ions and the release nucleotide incorporation in the PCR condition. While other argue that the presence of the RNA in DNA extracted is not major problem as this usually does not interfere with PCR or restriction digestion [9]. Because RNA is, by nature, transient and unstable unlike DNA. RNA is ubiquitously degraded with striking efficiency in all cells (Houseley et al 2009). Much of the RNA is cut by ribonucleases or RNAses that are released when the cells are broken open and the rest will not last in an environment outside the cell and will degrade anyways even without RNAse.

CONCLUSION:

In this study, three methods for used for isolating DNA from *Arachis hypogaea* were compared and analysed from the following perspectives: Quality and quantity of DNA, the purity of DNA acquired, and functionality. All the three methods compared in this study turned out to be suitable to extract DNA from *Arachis hypogaea.* In summary, the conclusions in this research are as follows:-

1. The yield of DNA from *Arachis hypogaea by* CTAB modified are significantly higher than those obtained by the CTAB and HipurA kit method.
2. The extraction method had a significant effect on the DNA yield and $OD_{260/280}$ ratio of control and SLF treated leaf tissue of *Arachis hypogaea*.
3. After evaluating the yield, purity, integrity, functionality among the three methods, the CTAB Modified method was considered an ideal protocol to isolate DNA from *Arachis hypogaea* by using control and SLF treated Leaves.
4. Besides, the quality and quantity of the DNA extracted by this method were high enough to perform hundreds of PCR-based reactions and also to be used in other DNA manipulation of RAPD Techniques.
5. The DNA was isolated from control and SLF treated leaves DNA samples were amplified with Ten random primers OPA-1 to OPA-10 (RAPD) and the polymorphic bands were identified .

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