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Full Length Research Paper

Protocol optimization for deoxyribonucleic acid (DNA) extraction from dried, fresh leaves, and seeds of groundnut (*Arachis hypogaea* L.)

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Consistent isolation of best quality deoxyribonucleic acid (DNA) from peanut (*Arachis hypogaea* L.) is particularly problematic due to the presence of phenolic compounds 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. In this study, we used fresh and dried leaves as well as seeds for optimization of high quality DNA isolation protocols from *A. hypogaea*. The DNA extracted with three different methods cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), and cesium chloride (CsCl) density gradient) were comparatively studied by polymerase chain reaction (PCR) analysis in terms of quantity and quality. High quality genomic DNA was obtained from fresh leaves by modified CTAB methods. The DNA obtained ranged from 1 to 2.5 ng/µl. DNA obtained by this method was strong and reliable showing its compatibility for simple sequence repeat (SSR) analyses. The SDS based methodology give large quantities of DNA contaminated with polysaccharides. Fresh leaves also gave best result in SDS method. The quantity and quality of DNA obtained was very poor in all the tested methods in case of dried leaf tissues. The current protocol will probably be useful for the extraction of high-molecular weight DNA from other plant materials containing large amounts of secondary metabolites and essential oils.

Key words: Polysaccharides, polyphenols, tannins, cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), cesium chloride (CsCl), secondary metabolites, SSR.

INTRODUCTION

Peanut is an important oil seed crops cultivated mainly in subtropical, tropical, and temperate regions globally (Proite et al., 2007). It is grown in more than 100 countries across America, Africa, and Asia on an area of about twenty five million hectares and nearly thirty five million tons were produced annually (FAO, 2007).

The development of consistent deoxyribonucleic acid (DNA) isolation protocol and polymerase chain reaction (PCR) analysis is the basic step for many biotechnological techniques, such as molecular markers and genetic engineering. Therefore, it has been used in many plant species, such as *Pinus radiate* (Claudia et al., 1998), *Arachis hypogaea* (Sharma et al., 2000), *Parkia timoriana*

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Abbreviations: DNA, Deoxyribonucleic acid; **CTAB,** cetyltrimethylammonium bromide; **SDS,** sodium dodecyl sulfate; **SSR,** simple sequence repeat.

(Robert et al., 2003), *Mucuna pruriens* (Padmesh et al., 2006), and chickpea (Dipankar et al., 2006). In general, it is difficult to extract and purify high-quality DNA from certain plants, because of the presence of large quantities of secondary metabolites (tannins, alkaloids, and polyphenols), polysaccharides and pro-teins. These compounds interferes DNA, thus degrading its quality and reducing yields (Katterman and Shattuck, 1983; Sarwat et al., 2006).

Consistent isolation of best quality DNA from peanut (*A. hypogaea* L.) is problematic, particularly, due to the presence of phenolic compounds 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. In this study, we report the rapid and reliable procedure for extracting good quality and high quantity of genomic DNA for PCR and molecular analysis.

METHODOLOGY

A total of 70 *A. hypogaea* accessions were collected from different research institutes, such as National Agricultural Research Centre (NARC), Bangladesh Agricultural Research Institute (BARI), and Ayub Agricultural Research Institute (AARI), Pakistan. Isolation of DNA was carried out from seeds, fresh and dried leaves sources of these samples. Three different DNA isolation protocols were comparatively studied in order to optimize the best suited protocol for *A. hypogaea*.

Reagents and chemicals

Tris-HCl (1.0 M; pH 8.0, 9.5); 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 8); 5.0 M NaCl; 3.0 M sodium acetate (pH 5.2); cetyltrimethylammonium bromide (CTAB; 20%); chloroform:isoamyl alcohol (24:1, v/v); and b-mercaptoethanol (AR grade). Modified CTAB extraction buffer: 0.1 M Tris-Cl (pH 9.5), 20 mM EDTA (pH 8), 1.4 M NaCl, CTAB (2%, w/v), and b-mercaptoethanol (1%, v/v) (added to the buffer just before use). Also with pure cold (-20°C) isopropanol, 70% ethanol, and absolute ethanol.

DNA extraction

We modified the pre-existing protocols described by Grattapaglia and Sederoff (1994) for the extraction of DNA from three different explants, that is, seeds, fresh and dried leaves of *A. hypogaea.*
One gram of leaves from each genotype were wiped clean with 70% ethanol. The leaves were grind in liquid nitrogen (LN) into fine powder with pestle and mortar. Fine powders were poured to 15 ml sterilized falcon tube containing extraction buffer and was properly mixed. The falcon tube was kept for 20 min in incubation bath at 65°C. After adding 15 ml sodium dodecyl sulfate (SDS) or (chloroform: isoamyl alcohol, 1:24) solution, it was shacked gently and was centrifuged at 8500 rpm for 30 min at room temperature. The supernatant was taken in a sterilized falcon tube and equivalent amount of isopropanol was added, after which it was kept at -20°C for 20 min, and was centrifuge at 8500 rpm at room temperature for 30 min. Then, the pellet was washed with 70% ethanol after removing the supernatant. DNA pellet was air-dried for about 1 h, and was dissolved in 1 ml ddH_2O and 1 µl RNase was added, and it was kept in the falcon tube at 37°C for 2 h. The amplified product on non-denaturing 1% agarose gel stained with ethidium bromide as described by Ferguson et al. (2004) for confirmation of DNA was visualized.

PCR analysis

DNA samples were diluted to 20 ng/µl for the microsatellite analysis. PCR analysis was carried out in Veriti 96-well thermal cycler (Applied Bio systems, CA) with Taq polymerase (MBI Fermentas). DNA sample were diluted to 20 ng/µl for the microsatellite analysis. PCR reaction were performed in 20 µl volumes containing 30 ng/µl genomic DNA, IX PCR buffer (MBI Fermentas) 1.6 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 U DNA polymerase (MBI Fermentas), and 10 pmol of each primer.

DNA isolation by other methods

Isolation of DNA from the same fresh as well as dried leaf samples was carried out by two other methods, SDS and cesium chloride (CsCl) density gradient. The DNA isolated by the other methods was subjected to analysis by agarose gel electrophoresis and PCR analysis were conducted to compare the quality and quantity of isolated DNA with modified CTAB method.

RESULTS AND DISCUSSION

The DNA extracted with three different methods (CTAB, SDS and CsCl density gradient) were comparatively studied by PCR analysis in terms of quantity and quality. High quality genomic DNA was obtained from fresh leaves by CTAB methods (Figure 1). The extracted DNA showed a reading in between 1.7 to 1.8 after calculating the ratio of absorbance, 260/280 nm. The DNA obtained ranged from 1 to 2.5 ng/µl. DNA obtained by this method was strong and reliable showing its compatibility for simple sequence repeat (SSR) analyses.

The SDS based methodology give large quantities of DNA contaminated with polysaccharides. Fresh leaves also gave best result in SDS method. The quantity and quality of DNA obtained was very poor in all the tested methods in case of dried leaf tissues (Figure 2).

The results show that both CTAB and SDS methods gave intact DNA, while that of CsCl density gradient gave sheared DNA bands. PCR analysis of SSR markers in genomic DNA obtained by CTAB method resulted in good quality amplification as compared to the other (Figure 3). Similarly, PCR analysis with DNA obtained by SDS methods was not consistent and the staining with ethidium bromide gave opaque bands indicating a very low concentration of amplified products.

The employment of modified CTAB methods for isolation of DNA from high level polysaccharides containing tissues by Sharma et al. (2000) supports our results. This method was appropriate for dry as well as fresh tissues and was experienced on soybean, chickpea seeds and wheat. When high concentration of CTAB (3% w/v) and

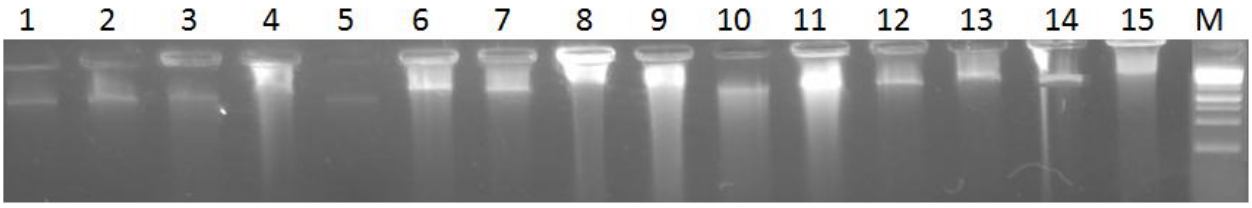

Figure 1. Ethidium bromide-stained agarose gel showing total DNA isolated CTAB method from fifteen samples of fresh leaves of (*A. hypogaea* L.). Lanes 1 to 15, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, Icgs-09, Icgs-17, Icgs-18, Icgs-114, and Icgs-38. Lane 16, DNA ladder.

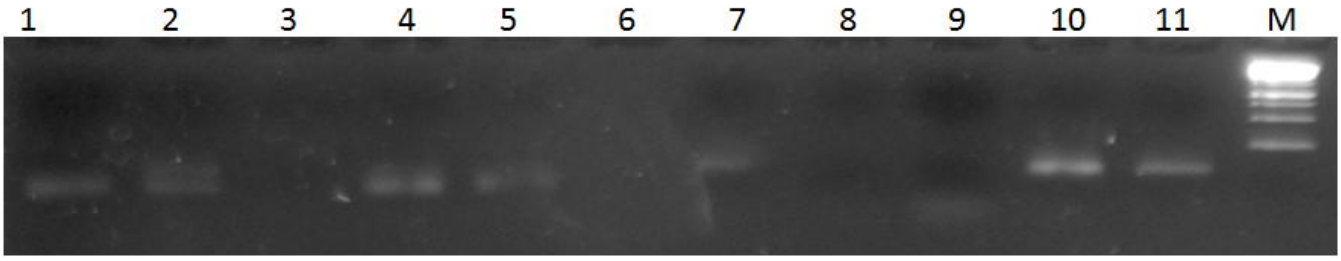

Figure 2. Ethidium bromide-stained agarose gel showing total DNA isolated by CTAB method from eleven samples of seeds powder of (*A. hypogaea* L.). Lanes 1 to 11, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, and Icgs-09. Lane 12, DNA ladder.

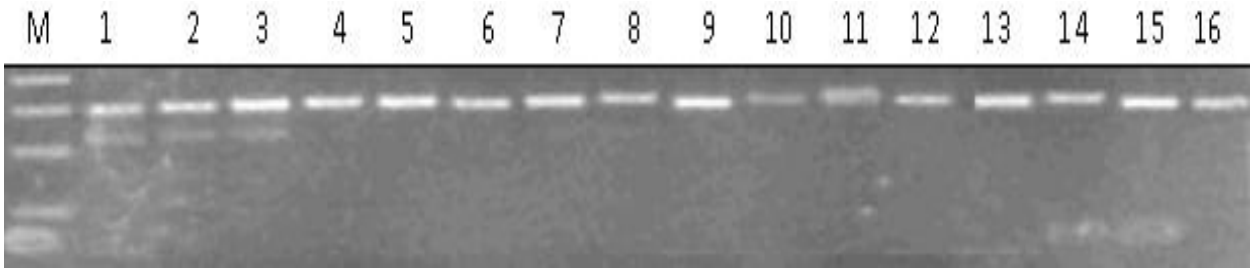

Figure 3. Ethidium bromide-stained agarose gel showing PCR-amplified products obtained from DNA isolated from fresh-leaf tissue by using ssr marker. Lane M, DNA ladder; lanes 1 to 16, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, Icgs-09, Icgs-17, Icgs-18, Icgs-114, Icgs-38 and Icg-48

sodium chloride (3 M) were combined in extraction buffer followed by washing with phenol: chloroform:isoamyl alcohol, majority of the polysaccharides can be successfully removed (Murray and Thompson, 1980; Paterson et al., 1993; Suman et al., 1999). Protein impurities can be successfully removed with double washing with phenol: chloroform:isoamyl alcohol (Dipankar et al., 2006). The use of phenol to remove CTAB polysaccharides complex formed earlier in the reaction was also reported in literature (Dipankar et al., 2006). Suman et al. (1999) successfully used the combination of high concentration of CTAB (3% w/v) and sodium chloride (3 M) in extraction buffer, followed by washing with phenol: chloroform:isoamyl alcohol for removal of polysaccharides. Protein impurities can be efficiently removed with double washing with phenol: chloroform:isoamyl alcohol (Dipankar et al., 2006). The polyvinylpyrrolidone (PVP) containing modified CTAB buffer was also used for extraction of DNA from *Tagetes minuta*. In SDS-based extraction methodology, SDS does not bind with protein, thus degrading the purified DNA (Deshmukh et al., 2007). Maliyakal (1992) stated that the PVP and polyphenols form a complex, allowing the polyphenols to be easily removable from the samples.

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