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An Easy, Quick and Cost Effective Method of High Quality DNA Extraction from Mungbean [*Vigna radiata* (L.) Wilczek] Without Liquid Nitrogen

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A B S T R A C T

Green gram is a widely cultivated pulse crop rich in protein, essential amino acids and vitamin-B. The quality and quantity of DNA are very important for amplification by PCR. Although quantity of DNA required per reaction in PCR is very low, quality is very crucial. Also, to carry out large number of PCR reactions for genotyping, a good amount of DNA is required. Presence of contaminants like phenols makes it difficult to get good quality DNA from mungbean. Thus, the present study was undertaken to obtain high quality and pure DNA in mungbean. The method involves extraction of DNA using a buffer (pH 8.0) containing 100mM Tris, 50mM EDTA, 500mM NaCl, 2%PVP, 2%CTAB and 0.2%β-mercaptoethanol followed by purification of DNA with chloroform and isoamyl alcohol and finally precipitation of DNA by sodium acetate and isopropanol. The method is suitable for extraction of DNA from small to large number of plant samples. DNA obtained through this protocol is of high quality and free of phenols which gave amplifying products in the PCR. Here, we developed a simple, fast, efficient, economical method for isolation of DNA from green gram without liquid nitrogen which could be stored for longer duration and does not require expensive chemicals such as proteinase K, liquid nitrogen etc.

Introduction

Green gram (*Vigna radiata* (L.) Wilczek) or mungbean is a self-pollinated widely cultivated leguminous crop in India. It belongs to the family Leguminaceae and subgenus Ceratotropis with diploid chromosome number (2n=2x=22) (Kang *et al.*, 2014). It is mainly grown in tropical Africa and Asia and several *Vigna* species have been domesticated in Asia. It is an important source of protein for the human population and soil health as it fixes atmospheric nitrogen.

High protein, easy digestibility and low flatulence make it widely acceptable to the people world over. The annual world production area of mungbean is about 5.5 million hectare of which about 90% is in Asia (Lambrides and Godwin, 2007). India is the largest producer of mungbean and occupies an area of 34.4 lakh hectares with a production of 1.60 million tonnes and productivity of 568 kg/ha (2015-2016). Genus *Vigna* have high amount of polyphenol, orthohydroxyphenols and polysaccharides.

These are powerful oxidizing agents to interfere with genomic DNA which can further inhibit the PCR amplification. Good quality and quantity of DNA is a prerequisite for various experiments of molecular biology. DNA purity is very important for amplification by PCR and to get clear and reproducible results.

For marker assisted selection, lesser number of PCR reactions are carried out and hence less amount of DNA is needed which further doesn't require long time storage (McCarthy and Berger, 2002). But, in case of QTL mapping or population studies of F_2, RILs etc. larger number of PCR reactions are to be performed and thus more amount of purified DNA is required as well as it need to be stored for a longer duration. Thus, an efficient, fast and easy method for DNA extraction is extremely required. Among the most commonly used basic plant DNA extraction protocols include Murray and Thompson (1980), Dellaporta *et al.,* (1983), Saghai Maroof *et al.,* (1984) and Doyle and Doyle (1990) along with many others that are modifications of the components of these protocols to suit a particular tissue type or downscaling them for miniprep. Most of these protocols are time consuming, comparatively expensive and require liquid nitrogen for grinding of samples (Sharma *et al.,* 2003; Allen *et al.,* 2006). Liquid nitrogen is usually expensive and difficult to procure in remote locations, a method not requiring its use would be more helpful. In addition to these basic protocols, several DNA isolation kits based on either anion exchange chromatography or silica gel membranes are available commercially. Though these kits are convenient and usually safe as they don't use hazardous reagents but their availability to certain developing countries and high cost can be limiting, especially when handing a large number of samples and considering experiments with limited financial resources Furthermore, in some instances, commercial kits have produced low DNA yields and variable quality (Chen *et al.,* 2006; Thomas and Dominic, 2015).

Although various protocols are available for DNA isolation in wheat (Stein *et al.,* 2001; Dellaporta *et al.,* 1983; McCarthy and Berger, 2002), *Cicer* (Chakraborti *et al.,* 2006), nodules of legumes (Krasova-Wade and Neyra, 2007) and in other plant species (Lange *et al.,* 1998). All of these protocols either involve elaborative and time consuming steps and use of expensive chemicals such as proteinase K, liquid nitrogen etc. On the other hand, some methods involve use of specific equipment designed especially for DNA isolation while others may be suitable for isolation of DNA from seed material and not for leaf tissue (McCarthy and Berger, 2002). In case of food legumes, presence of phenols and other contaminants offer difficulty in pipetting DNA and make DNA unamplifiable in PCR reaction by inhibiting *Taq* DNA polymerase. Therefore, a protocol is required which can provide high quality and pure DNA in green gram. Here, we describe a DNA extraction protocol suited for isolation of relatively pure DNA in sufficient amount from green gram that can be stored for a longer duration and gave amplified products for PCR reactions. The method is easy, rapid involving no liquid nitrogen and other hazardous and expensive chemicals like phenol, proteinase K etc.

Materials and Methods

Plant material

A total of 34 genotypes of mungbean were used for this study which included varieties from germplasm collection released from CCS HAU, Hisar, PAU, Ludhiana, Indian Institute of Pulses Research (Kanpur), some advanced breeding lines and exotic

germplasm lines (Table 1). Plants were grown in field conditions using standard agronomic practices. Young and healthy leaves of two week old seedlings were collected in ice box for DNA isolation and stored at -20°C until use.

Solutions and reagents

CTAB Buffer: The extraction buffer consisted of 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl, 2% (w/v) PVP-40,000, 2% CTAB. Just prior to use, add 0.2% (v/v) β-Mercaptoethanol to the extraction buffer and warm at 65°C in a water bath.

Chloroform: isoamyl alcohol (24:1) mixture

3 M sodium acetate (pH 5.8)

Isopropanol

70 % ethanol

TE buffer (10 mM Tris-HCl, 1 mM EDTA)

RNase A (10mg / ml)

DNA extraction protocol

3-4 young and healthy leaves were ground in 1ml CTAB extraction buffer without liquid nitrogen using a pestle and mortar. The mixture was transferred into eppendorf tubes with a spatula and 500 µl of extraction buffer was added further. It was incubated for 1 hr with occasional mixing by inversion in a water bath maintained at 65°C. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was collected into new eppendorf tubes. 500 µl of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inverting for 5 min followed by centrifugation at 10,000 rpm for 15 min at 4°C. The aqueous phase having DNA was carefully transferred to a fresh centrifuge tube avoiding the interphase. Again, equal volume of CI (24:1) was added to the tube and mixed gently followed by centrifugation. This step was repeated twice. DNA was precipitated by adding 1/10 volume of 3M sodium acetate and equal volume of ice-cold isopropanol followed by gentle mixing and kept at 4°C overnight. Next day, the samples were centrifuged at 10,000 rpm for 10 min and the resultant supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 10 min. This step was repeated twice. The pellet was air dried and dissolved in 150 µl TE buffer and stored at -20°C. 3 µl RNase A (10 mg/ml) was added to the tubes and were kept in water bath at 37°C for 1 hr.

DNA quantification

The yield of extracted DNA was analyzed on UV-VIS spectrophotometer by reading absorbance at 260 nm and purity was checked by taking the ratio at 260/280 nm. The isolated DNA was also analyzed on agarose gel electrophoresis. For gel preparation, 0.8 g of agarose was weighed and added to 100 ml of 1X TBE buffer to get final concentration of 0.8%. Agarose was dissolved properly by boiling in microwave oven and cooled to about 55°C. Then, 5 µ1 of ethidium bromide (10 mg/ml) was added to it and after mixing completely poured into the gel casting tray carefully without formation of any air bubbles and left for solidification for 20-30 min. Then, 2 µl DNA sample and 3 µ1 of 6X loading dye were mixed on a parafilm and loaded into the wells of the gel. λDNA of known quantity having concentration of *viz.*100 ng was also loaded to quantify the DNA samples. The gel was run at 80V, till the tracking dye migrated to the bottom of the gel. The DNA samples were visualized using a UV gel documentation system (Benchtop Lab

Equipment) and photographed and documented.

PCR assay

Amplification reactions were carried out in a volume of 20 µl reaction mixture containing 1U Taq DNA polymerase (G Biosciences), 1X PCR buffer (10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM dNTP mix, 0.5 µM forward and reverse primers, 5% DMSO, 50 ng of DNA. PCR cycles consisted of initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 50s, extension at 72°C for 2 min and a final extension at 72°C for 7 min. The amplified products were analyzed on 3.5% agarose gel and visualized under UV transilluminator and photographed using gel documentation system.

Results and Discussion

The isolation of high quality DNA is a crucial step in the field of plant molecular biology. Most DNA extraction methods are time-consuming, involve multiple steps, expensive chemicals and enzymes and use hazardous procedures of grinding plant tissue in liquid nitrogen (N_2) to break down the cell wall of plants (Sharma *et al.,* 2003) or freeze-drying (lyophilization) (Sperisen *et al.,* 2000). Procurement and storage of liquid nitrogen may be difficult for many laboratories and handling of the same is also difficult. Thus, any method which doesn't need liquid nitrogen can be more helpful. The need of liquid nitrogen during grinding step has been eliminated by many workers using soft tissues such as flower petals or young leaves (Khan *et al.,* 2004), cold and heat shock treatments, (Biswas and Biswas, 2011) alcohol fixation (Sharma *et al.,* 2003; Sharma *et al.,* 2010). (Duan *et al.,* 2015; Ibemhal *et al.,* 2015). Here, we developed an easy, fast, inexpensive method to isolate high quality genomic DNA from mungbean eliminating the use of hazardous chemical phenol and expensive reagents like proteinase K and liquid nitrogen. The yield of extracted DNA using this method ranged from 100.5-320.2 ng/µl and the ratio of $A_{260/280}$ varied from 1.78 to 2.00 indicating good quality DNA which is free from protein contamination (Table 2). Similar kind of yield and quality of DNA was also reported in other protocols (Henry, 1997; Sharma *et al.,* 2003, Sharma *et al.,* 2010). A ratio of absorbance 260/280 is used to assess the purity of nucleic acid and its accepted range is mainly ~1.8.

In addition, on analysis on agarose gel electrophoresis, it showed no visible DNA degradation or RNA contamination indicating good quality and quantity of extracted genomic DNA (Fig. 1). The good quality of DNA extracted in our study is comparable to various other studies where it was reported that good quality DNA can be isolated without using liquid nitrogen (Chandra and Tewari, 2009; Sharma *et al.,* 2010; Ferdous *et al.,* 2012).

For DNA extraction, the ground tissue is transferred to an extraction buffer which contains detergent to disrupt the membranes. Cetyltrimethyl ammonium bromide (CTAB) is commonly used for plant DNA extraction to disrupt the rigid cell wall. The extraction buffer also contains a reducing agent (β-mercaptoethanol) and a chelating agent (ethylenediamine tetraacetic acid, EDTA) which chelates the magnesium ions required for DNase activity. Thus, it inactivate nucleases that are released from the plant cell and causes serious degradation of the genomic DNA. Phenol extraction can be used to remove any traces of proteins and the genomic DNA can be precipitated using either ethanol or isopropanol. Precipitated DNA can be hooked out of the solution or collected by centrifugation. In the present study, the extracted DNA was free of phenols as the pellet obtained was clean and white to

pale yellow in colour while usually in pulses brown colored DNA pellet is obtained due to presence of phenols and the quality of DNA isolated was comparable to other such recent studies (Laxmi prasanna *et al.,* 2013; Healey *et al.,* 2014; Rawat *et al.,* 2016; Sajib *et al.,* 2017). Moreover, here we used young and tender leaves as they contain less phenol as compared to mature leaves which contain more phenol content and the DNA obtained is generally brown in color. Thus, young and tender leaves of approximately two week old seedlings are more ideal for DNA extraction in green gram. Phenolic compounds may also be released on disruption of plant tissues and these may interfere with subsequent uses of the DNA such as it may inhibit PCR. Contrary to it, the method described here is very efficient as the DNA obtained through this protocol was relatively pure which gave amplifying products in the PCR (Fig. 2). Horne *et al.,* (2004) reported that some contaminants such as phenols which inhibit PCR could not be removed with chloroform extraction, so to remove phenolic compounds during extraction, 2% polyvinyl pyrolidone (PVP) and 2% β-mercaptoethanol was added to the extraction buffer. β-mercaptoethanol is a reducing agent which helps in denaturing proteins by breaking the disulfide bonds between the cysteine residues and for removing the tannins and polyphenols present in the crude extract. In addition to saturated phenol, enzyme proteinase K is also used to remove proteins which however is again denatured by phenol via phenol chloroform extraction and moreover it is costly. Further in the present study to remove proteins, instead of using phenol/PCI (25:24:1), only CI (24:1) was used to get good quality DNA thus reducing cost as well as health risks associated with use of phenol.

Table.1 List of 34 genotypes of mungbean used in the study

S. No.	Genotype	Source	S. No.	Genotype	Source
1.	ML776	PAU, Ludhiana	18.	MH1142	CCSHAU, Hisar
2.	GP111	Germplasm line, Hisar	19.	Kopergaon	Akola, Maharashtra
3.	MH421	CCSHAU, Hisar	20.	SMH 99-1	CCSHAU, Hisar
4.	Pusa 1431	IARI, New Delhi	21.	BDYR-1	Exotic line
5.	MH 534	CCSHAU, Hisar	22.	2KM138	Coordinated program
6.	MH 565	CCSHAU, Hisar	23.	PM 827	PAU, Ludhiana
7.	Muskan	CCSHAU, Hisar	24.	ML 2037	IIPR, Kanpur
8.	Pusa 1532	IARI, New Delhi	25.	LGG 460	Guntur, AP
9.	IPM 9901-8	IIPR, Kanpur	26.	Pusa 1531	IARI, New Delhi
10.	Pusa 1542	IARI, New Delhi	27.	MH 98-1	CCSHAU, Hisar
11.	Pusa 1501	IARI, New Delhi	28.	Pusa 871	IARI, New Delhi
12.	Pusa 1502	IARI, New Delhi	29.	2KM 112	IARI, New Delhi
13.	Asha	CCSHAU, Hisar	30.	Pusa 1142	IARI, New Delhi
14.	SML668	PAU, Ludhiana	31.	MH 1157	CCSHAU, Hisar
15.	MH318	CCSHAU, Hisar	32.	MH 731	CCSHAU, Hisar
16.	Satya	CCSHAU, Hisar	33.	MH 810	CCSHAU, Hisar
17.	Basanti	CCSHAU, Hisar	34.	Pusa 9072	IARI, New Delhi

Table.2 Concentration and A_{260}/A_{280} ratio of extracted DNA from young leaves of 34 genotypes of mungbean

S. No.	Genotype	DNA Conc. (ng/µl)	$A_{260/280}$	S. No.	Genotype	DNA Conc. (ng/µl)	$A_{260/280}$
1.	ML776	152.4	1.85	18.	MH1142	300.4	1.83
2.	GP111	154.3	1.78	19.	Kopergaon	200.6	1.78
3.	MH421	200.1	1.90	20.	SMH 99-1	210.5	1.89
4.	Pusa 1431	140.3	1.83	21.	BDYR-1	320.2	1.84
5.	MH 534	130.4	1.80	22.	2KM138	250.6	1.83
6.	MH 565	128.2	2.00	23.	PM 827	220.3	1.79
7.	Muskan	125.1	1.86	24.	ML 2037	206.4	1.80
8.	Pusa 1532	135.3	1.79	25.	LGG 460	270.2	1.86
9.	IPM 9901-8	130.2	1.80	26.	Pusa 1531	300.7	1.78
10.	Pusa 1542	127.4	1.83	27.	MH 98-1	200.2	1.92
11.	Pusa 1501	150.2	1.78	28.	Pusa 871	150.4	1.80
12.	Pusa 1502	130.4	1.88	29.	2KM 112	100.5	1.91
13.	Asha	135.6	1.78	30.	Pusa 1142	230.1	1.80
14.	SML668	129.3	1.86	31.	MH 1157	100.6	1.98
15.	MH318	170.2	1.79	32.	MH 731	150.4	1.87
16.	Satya	160.4	1.80	33.	MH 810	120.3	1.82
17.	Basanti	190.2	1.81	34.	Pusa 9072	130.2	1.88

Fig.1 Analysis of genomic DNA extracted from various genotypes of mungbean on 0.8% agarose. Lane M- DNA, Lane 1-34 –various genotypes of mungbean

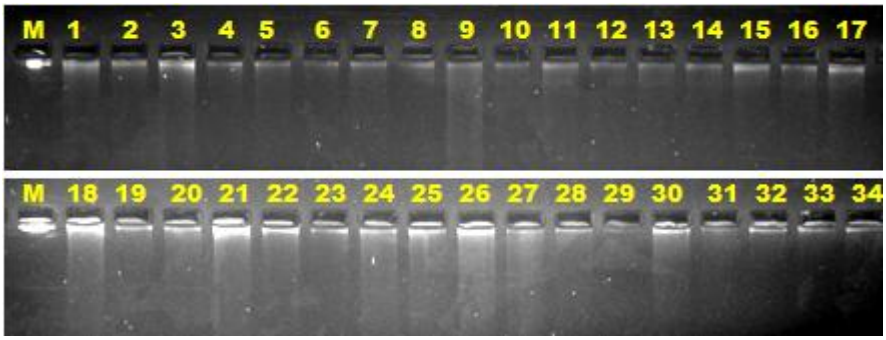
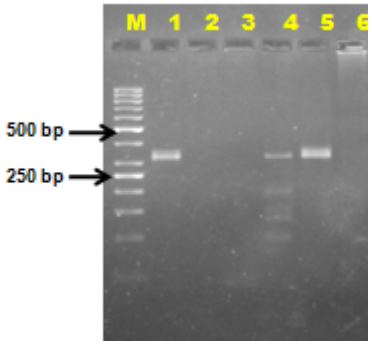


Fig.2 PCR amplification with extracted DNA using SSR marker on 3.5% agarose gel. Lane M-50 bp ladder, Lane 1-6 – DNA of various mungbean genotypes used for PCR



A salt such as sodium chloride is used in extraction buffer which aids in precipitation by neutralizing the negative charges on the DNA so that the molecules can come together. At lower NaCl concentrations (<0.4 M), DNA and CTAB form an insoluble complex (Murray and Thompson, 1980) which is separated from the residual soluble proteins, polysaccharides and other molecules by centrifugation. Similar low salt/CTAB strategies have been used to collect DNA in mungbean (*Vigna radiata*) (Murray and Thompson, 1980), orpine (*Sedum telephium*) (Barnwell *et al.,* 1998), cotton (*Gossypium* spp.) (Zhang and Stewart, 2000) and rice (Vibhuti *et al.,* 2015). Further, chloroform is required to remove major protein, phenolics and cell debris contaminates. The DNA is precipitated using equal volume of isopropanol and diluting the nucleic acid with a monovalent salt and mixing gently and the pellet is collected by centrifugation. The salts are removed by washing with 70% alcohol.

The most commonly used salts include sodium acetate pH 5.2 (final volume 0.3M), sodium chloride (final concentration 0.2M), ammonium acetate (2-2.5M), lithium chloride (0.8M) and potassium chloride. Ethanol (twice the volume) or isopropanol (equal volume) are the standard alcohols used for nucleic acid precipitation. It is important that DNA is not sheared, for this reason the DNA should not be vortexed or pipetted repeatedly using a fine tipped pipette and all manipulations should be as gentle as possible. Further, keeping the reactions cold during isolation steps can minimize their effects and improve the quality of DNA. After RNAase treatment, people generally follow purification steps by performing CI extractions which can reduce DNA quantity. Here, no purification steps were carried out further after RNAse treatment which saves time and good quality DNA was also observed which was directly used for PCR amplification and good intensity of bands was observed.

In summary, here we describe a simple, safe, fast and cost efficient CTAB DNA extraction protocol without liquid nitrogen which provides high quality pure DNA from mungbean which generally contain high concentration of phenolic compounds. Thus, it gives sufficiently pure DNA, which may be suitable for carrying out large scale marker analysis and genotyping reducing time and cost and moreover the same may be preserved for a longer duration.

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