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Research article

STANDARDIZATION OF DNA EXTRACTION PROTOCOL IN GREENGRAM (*Vigna radiata* (L.) Wilczek)

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ABSTRACT: Green gram is a widely cultivated pulse crop rich in protein, high in vitamin-B content and essential aminoacids. It is easily digestable and low flatulence produced crop. The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of genotyping. Especially, DNA purity is extremely crucial for obtaining clear and discriminate patterns. DNA extraction from Green gram is difficult due to presence of contaminants such as phenols. Therefore, the present study was under taken to obtain high quality and pure DNA in Green gram. With few modifications four different DNA extraction protocols were tried in the present study to obtain high quality and pure DNA *viz.,* (i) Doyle and Doyle (1987), (ii) Method of Murray and Thompson (1980), (iii) Porebski *et al.*(1997), and (iv) Lin *et al.* (2001). Out of the four methods tried for DNA extraction, the method of Lin *et al.* (2001) was found most efficient, as the DNA obtained through this protocol was relatively pure which gave amplifying products in the PCR. The genotype used for the standardization was MGG -361.

Key words: Green gram, DNA extraction, Standardization

INTRODUCTION

Green gram (*Vigna radiata* (L.) Wilczek) or mungbean is a self pollinated widely cultivated pulse crop in India. *Vigna* species are an important source of protein for people, particularly in tropical Africa and Asia and several *Vigna* species have been domesticated in Asia. High protein, easy digestibility and low flatulence production of green gram made the crop more acceptable by the people world over. In India, green gram occupies an area of 34.4 lakh hactares with a production of 14 lakh tones and with a productivity of 407 kg^{-1}ha (2010-2011). In Andhra Pradesh it is cultivated in 2.84 lakh hactares with a production of 1.34 lakh tonnes and with a productivity of 471 kg^{-1}ha. (2011-2012). The quality and quantity of DNA used for amplification by PCR is the key to reproducible results and success of genotyping. Especially, DNA purity is extremely crucial for obtaining clear and discriminate patterns. Genus *Vigna* have high amount of polyphenol, orthohydroxyphenols and polysaccharides. These are powerful oxidizing agents to interfere with genomic DNA. Some varieties are recalcitrant to inhibit the PCR amplification. DNA isolation protocols for *Cicer* have been reported by Chakraborti *et al*. (2006) and from nodules of legumes have been reported by Krasova-Wade (2007). Presence of phenols and other contaminants offer difficulty in pipetting DNA and make DNA unamplifiable in PCR reaction by inhibiting *Taq* DNA polymerase. Therefore, the present study was taken to obtain high quality and pure DNA in green gram.

MATERIALS AND METHODS

Chemicals used: (as per the protocol)

1) Extraction buffer, which is made by mixing of 1.25 mM Nacl, 100 mM Tris cl, 25 mM EDTA, 2% CTAB, 0.2% β-mercapto-ethanol, 3% Poly Vinyl Pyrolidine and required amount of distilled water.
2) Tris EDTA (TE) buffer : 10 mM Tris Cl, 1 mM EDTA
3) 95% cold ethanol, 70% Ethanol (70ml ethanol+30ml Milliq water) and absolute ethanol.
4) Ice cold Isopropanol.
5) C.I: Chloroform: Iso amyl alcohol, 24: 1v/v). 6) P.C.I (Phenol: Chloroform: Iso amyl alcohol, 25: 24: 1).
7) 5 M NaCl 8) RNase A (10 mg/ml). 9) 2 M Sodium acetate.

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Four protocols (enlisted below) were followed for standardization of DNA isolation from mungbean. For this purpose radical tips of 0.3 mm length and young leaves of one week old seedlings of mungbean were used. Of the 4 protocols used, the DNA extraction method described by Lin *et al.* (2001) could give good quality DNA enable to PCR analysis. The genotype used for the standardization was MGG -361. List of 19 mungbean genotypes used to confirm the Lin *et al.* (2001) protocol is furnished in table 1.

The details of four DNA extraction protocols tried in the present study are given below:

1. CTAB method of DNA extraction by (Doyle and Doyle 1987)
2. DNA extraction by Murray and Thompson (1980) with few modifications
3. DNA extraction by Porebski *et al.* (1997) with few modifications
4. DNA extraction by Lin *et al.* (2001) with few modifications

Table: 1. List of 19 mungbean genotypes used to confirm the Lin *et al.* (2001) protocol.

S.No.	GENOTYPE	SOURCE
1	GIVT -203	IIPR, Kanpur
2	KM – 8-657	IIPR, Kanpur
3	EC – 19515	NBPGR, New Delhi
4	PDM – 11	IIPR, Kanpur
5	RM – 9-126	IIPR, Kanpur
6	RM -9 -128	IIPR, Kanpur
7	LGG – 538	Lam, ANGRAU
8	Km – 8 -651	IIPR, Kanpur
9	MGG – 335	Madhira, ANGRAU
10	KM – 8-666	IIPR, Kanpur
11	NM – 1	NDUAT, Faizabad
12	MGG-361	Madhira, ANGRAU
13	MGG – 349	Madhira, ANGRAU
14	Pusa – 9531	IARI, New Delhi
15	MGG – 353	Madhira, ANGRAU
16	MGG – 351	Madhira, ANGRAU
17	KM-8-605	IIPR, Kanpur
18	KM-8-664	IIPR, Kanpur
19	UPM -84-178	IIPR, Kanpur

CTAB method of DNA extraction (Doyle and Doyle 1987)

Young leaf samples from 1 week old seedlings, were collected directly in eppendorf tubes were ground into fine powder with the help of micro pestle by freezing in liquid Nitrogen which facilitates easy grinding**.** To the powder 400 µl CTAB extraction buffer with 0.2% β- mercapto ethanol was added and the sample tubes were kept in water bath at 60 ^{0}C for 30-60 min with occasional and proper mixing after every 5-10 minutes, such that to see not to form any clumps at the bottom. Tubes were removed from water bath and then contents were allowed to cool to room temperature. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and mixed thoroughly by gentle inversion for 15 minutes by keeping in rotator @ 20 rpm (Rotospin, Tarsons) until clear separation of three layers is attained. After that contents were centrifuged at 12000 rpm for 12 minutes at 24^{0}C temperature. The clear aqueous phase (supernatant) was carefully pipetted out into new tubes. The chloroform: isoamyl alcohol (24:1 v/v) step was repeated twice to remove the organic contaminants in the supernatant. To the supernatant, cold isopropanol of about 0.5 to 0.6 volume (2/3rd of pipette volume) was added. The contents were mixed gently by inversion. Subsequently the tubes were centrifuged at 12000 rpm for 12 min at 24^{0}C temperature to pellet out DNA. The supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 4-5 min. This step was repeated twice. The tubes were allowed for air drying until the pellet gets completely dried and then the pellet was dissolved in 40 µl TE buffer. DNA was stored in -20^{0}C for further use. Results of CTAB method of DNA extraction by (Doyle and Doyle 1987) were furnished in (fig.1).

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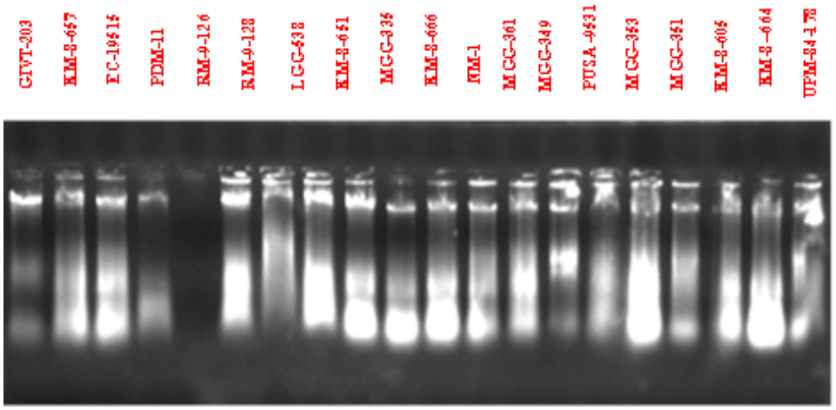

Figure-1: Genomic DNA isolation by Doyle and Doyle (1987) method with few modifications

DNA extraction method of Murray and Thompson (1980) with few modifications

15-20 radical tips were ground to fine powder in liquid nitrogen using a pestle and mortar. To the powder 500 µl of extraction buffer was added. Suspended the clumps with a spatula then transferred into eppendorf tubes and incubated for 1 hr with frequent mixing in a water bath maintained at 65°C. 500 µl of chloroform: iso-amyl alcohol (24:1) was added and mixed gently @ 20 rpm speed for 10 min. The tubes were centrifuged at 13000 rpm for 15 min and the supernatant was collected into new eppendorf tubes. This step was repeated twice.

DNA was precipitated by adding 2/3rd volume of ice-cold Isopropanol followed by gentle mixing and incubated for 1 hr at -20°C followed by centrifugation at 13000 rpm for 15 min at 4°C. The resultant supernatant was discarded and DNA pellet was washed with 1 ml of 70% ethanol then allowed for air drying and was dissolved in 50µl of TE buffer. After that the DNA was transferred to new eppendorf tube using half cut tip (to avoid DNA shearing), and 3 µl RNase (10 mg/ml) (pre-boiled) was added to the DNA solution and incubate at 37^{0} C for 1 hr. Re-precipitated the DNA by adding 0.1 volume of 5 M ammonium acetate and two volumes of 100% ethanol followed by gentle mixing and stored at -20^{0} C for 1 hr. The samples were centrifuged at 13000 rpm for 10 min and the supernatant was drained out. 500 µl of 70% ethanol was added to the DNA pellet and centrifuge at 13000 rpm for 10 min. The supernatant was drained carefully and the DNA pellet was air dried over night. The DNA pellet was dissolved in 50 µl of TE buffer.Results of DNA extraction method of Murray and Thompson (1980) with few modifications furnished in (fig 2).

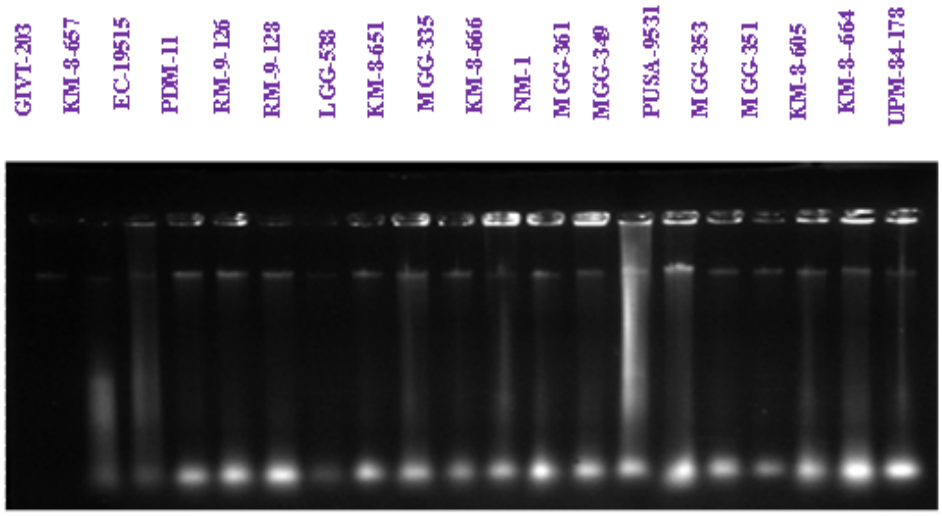

Figure-2: Genomic DNA isolation by Porebski et al (1997) method with few modifications

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DNA extraction method of Porebski *et al.* (1997) with few modifications

0.5 gm of cotylidonary tips were grounded using mortar and pestle in liquid nitrogen until finely powdered. Frozen ground tissue was transferred to eppendorf tubes. 500 µl of pre heated (60°C) extraction buffer was added. The contents were mixed by gentle inversion and incubated in a water bath maintained at 60 ^{0}C (with intermittent gentle shaking) for 25-60 min. The tubes were removed from water bath and cooled to room temperature for 4-6 min. 500 µl of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion to form an emulsion. After mixing thoroughly they were centrifuged at 3000 rpm for 20 min in a centrifuge at room temperature. The top aqueous phase was transferred carefully to new eppendorf tubes using wide-bore pipette tip. Chloroform-isoamyl alcohol extraction step was repeated again to remove cloudiness in aqueous phase. 1/2 volume of 5 M NaCl was added to the final aqueous solution recovered and mixed well. Two volumes of cold (-20°C) 95% ethanol was added and mixed by gentle inversion and placed in -20°C for 10 min to accentuate precipitation. The solution was left at 4-6°C to precipitate overnight. Centrifugation was done at 3000 rpm for 6 min. Supernatant was discarded and pellet was washed with cold (0-4°C) 70% v/v ethanol. The pellet was dried and dissolved in 40 µl of TE buffer and left overnight at 4-6°C. 3 µl RNAse A (10 mg/ml) was added and incubated at 37 ^{0}C in water bath for approximately 1 hr, the tubes were removed and 3 µl proteinase K (1 mg/ml) was added and incubated at 37^0 C for 15-30 min. 150 µl of phenol and 150 µl of chloroform was added to each eppendorf tube. They were rotated for a brief period and centrifuged at 14,000 rpm for 10-15 min. Upper layer was collected in new eppendorf tube. 1/10th volume 2 M Sodium acetate and 2 volumes of absolute ethanol were added and mixed gently. The samples were left overnight in -20^0 C. Samples were centrifuged at 14,000 rpm for 10-20 min. Later the samples were drained and washed with 70% v/v ethanol and the tubes were air dried. 40 µl TE buffer was added and allowed time for complete re-suspension. Results of DNA extraction method of Porebski *et al.* (1997) with few modifications furnished in (fig 3).

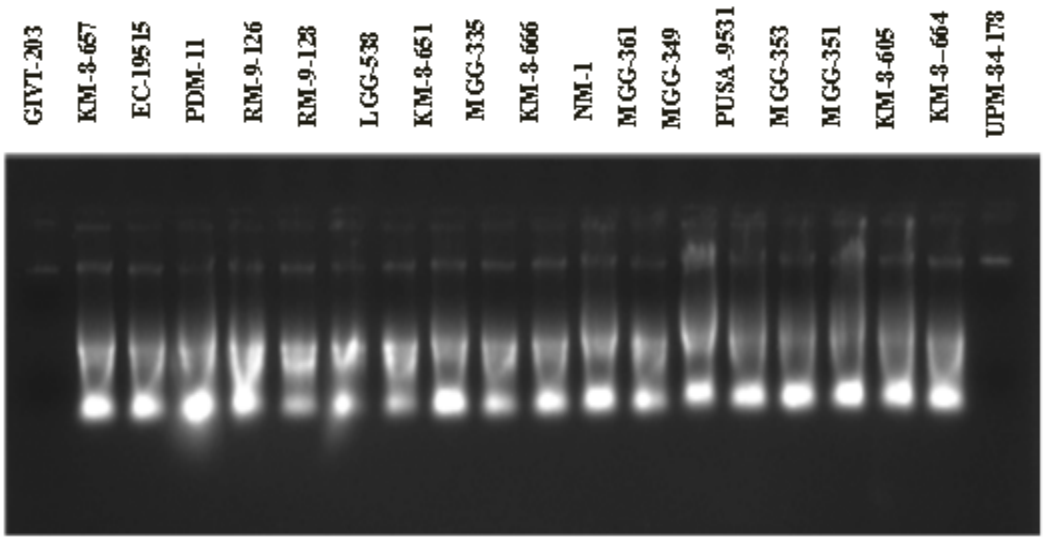

Figure-3: Genomic DNA isolation by Murray and Thompson (1980) method with few modifications

DNA extraction by the method of Lin *et al.* (2001) with few modifications

Collected leaf samples /root tips of 0.3 mm length from 15-20 germinated seeds of greengram were grounded into fine powder with the help of micro pestle in liquid nitrogen**.** 400 µl CTAB extraction buffer with 0.2% β-mercaptoethanol was added and the sample tubes were kept in water bath at 65^{0}C for 30 min with occasional and gentle but proper mixing after every 5-10 minutes. Tubes were removed from water bath and then contents were allowed to cool to room temperature. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) was added and mixed thoroughly by gentle inversion for 15 minutes by keeping in rotator @ 20 rpm until clear separation of three layers is attained. After that contents were centrifuged at 12000 rpm for 12 minutes at 24°C temperature. The clear aqueous phase (supernatant) was carefully pipetted out into new tubes. To the supernatant cold isopropanol of about 0.5 to 0.6 volume (2/3 rd of pipetted volume) was added. The contents were mixed gently by inversion. Subsequently the tubes were centrifuged at 12000 rpm for 12 min at 24°C temperature to pellet out DNA.

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The supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 4-5 min. This step was repeated twice. The tubes were air dried and then the pellet was dissolved in 40 µl TE buffer and stored at -20°C over night. 3 ul RNase A (10 mg/ml) was added and the sample tubes were kept in water bath at 37 ^{0}C for 1 hr. Tubes were removed from water bath and then contents were allowed to cool to room temperature. 400 ul of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added and mixed thoroughly by gentle inversion for 15 minutes by keeping in rotator @ 20 rpm until clear separation of three layers is attained. After that contents were centrifuged at 12000 rpm for 12 minutes at 24°C temperature. The clear aqueous phase (supernatant) was carefully pipetted out into new tubes. To the supernatant cold isopropanol of about 0.5 to 0.6 volume (2/3 rd of pipetted volume) was added. The contents were mixed gently by inversion. Subsequently the tubes were centrifuged at 12000 rpm for 12min at 24°C temperature to pellet out DNA. The supernatant was discarded gently and the DNA pellet was washed with 70% ethanol and centrifuged at 10,000 rpm for 4-5 min. This step was repeated twice. The tubes were allowed for air drying until the pellet gets completely dried and then the pellet was dissolved in 40 µl TE buffer. DNA was stored in -20°C for further use. Results of DNA extraction method of Lin *et al.* (2001) with few modifications furnished in (fig 4).

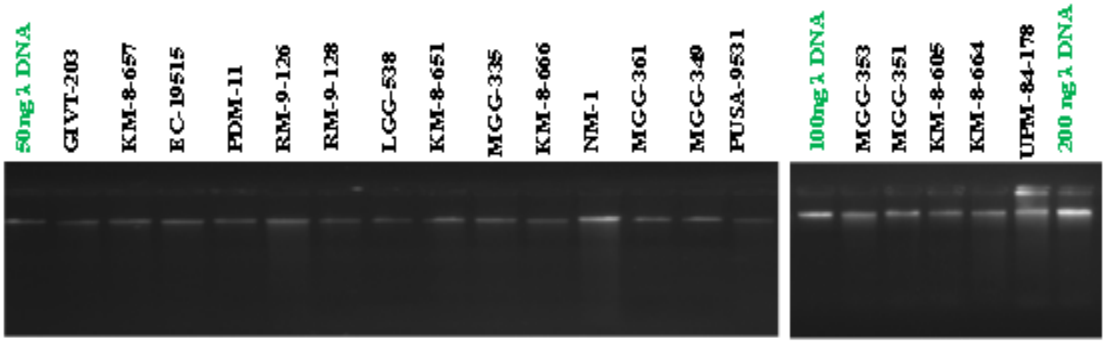

Figure-3: Genomic DNA isolation by Lin et al (2001) method with few modifications

DNA quantification

0.8 gm of agarose was weighed and added to 100 ml of 1X TAE buffer to get final concentration of 0.8%. Agarose was dissolved properly by boiling in microwave oven and then cooled to room temperature. Just before complete cooling 3 µ1 of ethidium bromide (10 mg/ml) was added to it and mixed thoroughly. Gel casting tray was prepared by fitting the comb properly into the gel casting tray in such a way that 2mm gap was maintained between the bottom of the tray and comb tip. The melted cool agarose (with ethidium bromide) was poured into the casting tray carefully without formation of any air bubbles and left for solidification for 20-30 min. After proper solidification of the gel, the casting tray along with gel was placed horizontally in electrophoresis apparatus and the comb from casting tray was removed carefully. Required amount of 1X TAE solution was added to the apparatus such that the gel was completely immersed. DNA samples for loading were prepared by placing of 2 µ1 of 6X loading buffer on parafilm, to which further 2 µl of DNA was added. The DNA samples from parafilm were gently loaded in to the wells of the gel. λ DNA of known quantity having concentration of *viz.*, 50 ng, 100 ng and 200 ng was loaded in order to quantify the DNA samples. The gel was run at 70V, till the tracking dye migrated to the bottom of the gel. The electrophoresed DNA samples were visualized using a UV gel documentation system (Syngene G BOX HR, UK) and the same was photographed and documented.

RESULTS

Four different DNA extraction protocols were tried with few modifications (composition and components of extraction buffer) to obtain high quality and pure DNA. Out of the four methods tried for DNA extraction, the method of Lin *et al.* (2001) was found most efficient, as the DNA obtained through this protocol was relatively pure which gave amplifying products in the PCR.

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