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ISSN: 0975-928X

Volume: 10

Number:1

EJPB (2019) 10(1):167-176

DOI: 10.5958/0975-928X.2019.00020.6

https://ejplantbreeding.org

Electronic Journal of Plant Breeding, 10 (1): 167 – 176 (Mar 2019) DOI: 10.5958/0975-928X.2019.00020.6
ISSN 0975-928X

Research Article

An improved method for rapid isolation of DNA and RNA from leaves, flowers and roots of blackgram [*Vigna mungo* (L.) Hepper] for detection of begomovirus infection and RT-PCR.

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(Received: 04 Jan 2019; Revised: 12 Feb 2019; Accepted: 13 Feb 2019)

Abstract
Blackgram is an important pulse crop grown for its protein rich seeds. Its tissues are rich in polyphenols which hampered pure nucleic acid extraction and thus use of molecular techniques in breeding programme. In this study, a well established DNA extraction method for legumes, Gem-CTAB method has been modified to isolate PCR compatible DNA from blackgram in a quick and economical way from a very small tissue. The amplification rate of DNA extracted through modified Gem-CTAB method was found to be high compared to Gem-CTAB method for begomovirus specific genes. While for RNA isolation, among different protocols, column based kit method followed by lithium chloride precipitation yielded high quality RNA for leaves compatible with RT-PCR. For flowers and roots, kit method alone or with its modifications yielded pure RNA. The above modified nucleic acid extraction method yielded PCR compatible DNA and RNA from phenolics rich blackgram tissues.

Keywords
DNA, RNA, Blackgram, Modified Gem-CTAB, TRIzol reagent

Introduction

Blackgram [*Vigna mungo* (L.) Hepper] is an important pulse crop, grown in the Indian subcontinent and other Asian countries for its protein rich seeds. The major constraints in blackgram production are considered to be biotic and abiotic stresses, which cause huge yield losses. Among the biotic stresses, the damage caused by yellow mosaic disease (YMD) is very prominent.YMD is caused by different strains of at least three different viral species belonging to the genus Begomovirus: *Mungbean yellow mosaic virus* strains genetically most similar to a strain from urdbean (MYMV-Urdbean) are predominant in North India. Strains most similar to MYMV-*Vigna* are predominant in South India and *Mungbean yellow mosaic India virus* (MYMIV) strains are predominant in Eastern India (Nair *et al*., 2017). Two cryptic species of whitefly (*Bemicia tabaci*) that transmit the disease have been found (Nair *et al*., 2017). Asia II 1 is prevalent in Northern India, while Asia II 8 is prevalent in Southern India (Nair *et al*., 2017). Breeding for yellow mosaic disease resistance needs identification of suitable resistance gene(s) against different viral strains /white fly species. Plant viruses evolve rapidly with time and virus resistant genotypes of crops are generally specific to particular viral strains. Hence, there is a need for identification of viral strains in a geographical region to develop varieties resistant to target virus strains and for early detection of virus for timely prevention of disease spread.

Extracting high-quality nucleic acids from legume species is cumbersome because legume crops especially blackgram are rich in polysaccharides, polyphenols and other secondary metabolites (Babu *et al*., 2014) that cause hindrance in nucleic acid extraction. Concentration of these compounds increase in plants under biotic and abiotic stresses, such as pathogen infection or drought (Chaves *et al*., 2003; Logemann *et al*.,1987; Lopez and Gomez 1992) that make nucleic acid extraction more cumbersome. Secondary metabolites interfere in nucleic acid extraction such as polyphenols being sensitive to oxygen, get oxidized to quinones and covalently bind to nucleic acids (Loomis, 1974). Polysaccharides and secondary metabolites co-precipitate with RNA owing to their similar physicochemical properties (Sharma *et al*., 2003; Key *et al*., 2012). These contaminants make resuspension of precipitated RNA difficult and interfere with absorbance based quantification, enzymatic manipulations and electrophoretic migration (Wilkins and Smart, 1996). In addition, these metabolites in RNA elute may interfere with highly sensitive downstream applications such as polymerase chain reaction (PCR), sequence

expressed tag marker assisted polymorphism, cDNA library construction, and microarray hybridization or they may also completely degrade RNA (Babu *et al*., 2014).

Since long, numerous methods have been developed to extract pure nucleic acids from legumes and with time several modifications have been incorporated to existing one for improvement. In earlier times, begomoviruses were detected and differentiated by serological methods which were often misleading (Swanson *et al*., 1992) and replaced by PCR based detection (Rouhibakhsh *et al*., 2008). Rouhibakhsh *et al*., (2008) demonstrated the suitability of CTAB protocol with ten different modifications reported by other workers (Dellaporta *et al*., 1983; Lodhi *et al*., 1994; Birnboim and Doley, 1979; Jose and Usha, 2003) in extracting high quality DNA from mungbean yellow mosaic India virus (MYMIV) infected blackgram leaves. Among the ten modifications, Gem-CTAB method was found to be the best with PCR compatibility for 13 out of 16 samples and also validated using 136 samples from different species with 72% validation rate. But three blackgram samples were PCR incompatible and some blackgram samples did not show amplification for all virus specific primers used in the study (Rouhibaksh *et al*., 2008). In case of RNA extraction from blackgram leaves, very few methods have been reported such as TRIzol reagent followed by RNeasy Plant minikit purification for young leaves (Kundu *et al*., 2015), and sucrose-sodium chloride (NaCl) and Tris-Hydrochloride (Tris-HCl) based extraction buffer protocol followed by Lithium chloride (LiCl) precipitation for blackgram leaves, flowers and roots (Babu *et al*., 2014). The above methods suggest that pure RNA cannot be isolated solely based on TRIzol or RNA extraction kit or sucrose-sodium chloride extraction buffer. Two or more different protocols in combination were deployed to extract pure RNA. TRIzol reagent has been mostly used along with additional purification protocols and sucrose-sodium chloride method required large quantities of tissue samples and other reagents including LiCl precipitation at 4˚C for 20 hours which is time consuming and associated with risk of RNA degradation due to RNase contamination. Therefore, the present study aimed at standardizing a best suited protocol for both DNA and RNA isolation from different blackgram tissues and also demonstrating the isolated DNA for PCR amplification with begomovirus specific genes and RNA for downstream application in RT-PCR.

Material and Methods

DNA was isolated from 40 days old matured leaves of 14 blackgram genotypes (WBG-57, T-9, TU-43-1, KU96-3, MDU-1, LBG-611, Co-6, TAU-1, Pusa-3, LBG587, PantU-31, PU-19, UH-86-321 and KU96-7) with YMV symptoms by Gem-CTAB (Rouhibaksh *et al*., 2008) and Modified Gem-CTAB(present study) methods. Plants were grown in open field condition for allowing natural YMV infection by whiteflies in Experimental Field Facility of Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai. Normal cultural practices were followed, with a highly susceptible cultivar to YMV was used as an insect refuge after every five rows. Under field conditions, resistant plants did not show any yellowing of leaves or pods during the growth period, while susceptible plants showed yellowing on the leaves and pods.

In modified Gem-CTAB method, approximately 20 mg. of fresh leaf tissues were ground to fine powder in liquid nitrogen using pre-chilled mortar and pestle and transferred to 0.5 ml. eppendorf tubes. DNA was extracted following the same Gem-CTAB protocol with few modifications as follows : 1)200µl of Gem-CTAB extraction buffer (100mM Tris–HCl pH 8, 10mM EDTA,1.4–2.0MNaCl, 2% CTAB and 2–5% β-mercaptoethanol) was added to 0.5 ml. tubes and incubated for lysis at 95˚C for 10 min. in thermal cycler (Eppendorf, Hamburg, Germany), 2) DNA was precipitated with cold isopropanol at -80˚C for 10 min.

DNA extracted by both the methods were subjected to PCR amplification using primers based on *actin* gene sequence and begomovirus specific genes, MYMIV-MP and MYMIV-NP primers (Table 1). Amplifications were performed in 25µl reaction volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.08% Nonidet P40, 0.2 mM each of dNTPs, 1.5 pmoles of each forward and reverse primers, 0.5 unit of *Taq* polymerase (Biotech Rabbit) with 75ng. of genomic DNA in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). PCR was done under touchdown thermal cycling comprising of single step of initial denaturation at 95°C for 3 min, 10 cycles of denaturation at 94°C for 30 s, 56°C for 30 s, 72°C for 60 s with lowering of annealing temperature at every cycle by 1°C followed by 35 cycles of denaturation at 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and final extension at 72°C for 10 min.Amplified products were electrophoresed at 80 V in 2% agarose gel in 1X Tris-borate- EDTA

(TBE) buffer and documented using a gel documentation system (Syngene, U.K.). Size of the amplicons were estimated in comparison with 100 bp DNA marker (MBI Fermentas, Germany).

Mature leaves, flowers and roots free from nodules were collected after 26, 40 and 28 days respectively and flash frozen in liquid nitrogen immediately and preserved at -80°C for later use. Molecular biology grade chemicals and millipore water were used to prepare solutions. Pipette tips, mortars and pestles were soaked overnight in 0.1% DEPC-treated distilled water, oven dried and autoclaved to make them RNase free. RNA was extracted from100 mg. of blackgram leaves, flowers and roots separately by 2 different methods with modifications,1) Silica column kit based method and 2) TRIzol based single step isolation method. In Silica column kit based method, total RNA was isolated using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) following the manufacturer's protocol with following modifications.:1a) double the amount of β-mercaptoethanol in lysis buffer, 1b) twice on-column washing with wash solution 1 and 2 provided in the kit and1c) LiCl (8M) precipitation of RNA elute at -80 ˚C for 10 min. While in TRIzol based method, total RNA was isolated using TRIzol™ reagent (Invitrogen, Carlsbad, CA) following the protocol (Chomczynski and Sacchi 1987) with following modification. Precipitation of RNA with LiCl (8M) at – 80˚C for 10 min instead of overnight incubation at – 20˚C .

Quantity and quality of DNA and RNA extracted from different methods in this study were assessed through A260/230 and A260/280 ratios obtained by Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA).Quality and integrity of DNA and RNA were also analysed by electrophoresis in 0.8 % and 1.25% agarose gel respectively followed by staining with ethidium bromide and documented using a gel documentation system (Syngene, U.K.). RNA samples were treated with DNase-I (Sigma-Aldrich, USA) and cDNA was synthesized with 2µg of total RNA using a PrimeScript™ RT-PCR Kit (Clontech, USA) in a 20 µl reaction volume using oligo (dT)18 primers according to manufacturer's manual. Reverse transcription programme comprised of 2 different thermal conditions: first incubation at 65 ˚C for 5min of mixture containing RNA, dNTPs, oligo (dT)18 primers and second incubation at 37 ˚C for 45 mins with addition of reaction buffer, RNase inhibitor, RT enzyme and nuclease free water. The PV18S primer pair amplifying ribosomal RNA gene from *Phaesolus vulgaris* was used for PCR (Table 1). PCR reaction for cDNA was carried out in a 25 µl reaction volume with same composition and cycling conditions as described for *actin* gene amplification earlier. Differential expression analysis was also done for five blackgram specific genes (Table 1) using cDNA synthesised from RNA isolated from leaves through kit+LiCl method.

Results and Discussion

Evaluation of DNA extracted by different methods for various parameters with their respective tissue weight is presented in Table 2. Modified Gem-CTAB method involving incubation at 95˚C yielded triple the amount of total nucleic acid in comparison to Gem-CTAB method involving incubation at 65 ˚C. Crude DNA samples were appeared as single band on 0.8% agarose gel. Both A260/280 and A260/230 ratios were found to be high for modified Gem-CTAB method (2.00, 2.17) compared to Gem-CTAB (1.97, 2.05) (Table 2). This indicates that high temperature and reduced lysis incubation time do not affect integrity and purity of DNA samples. DNA extracted from all 14 genotypes by Gem-CTAB and modified Gem-CTAB protocols showed amplification for *actin* gene (Fig. 1). For Gem-CTAB method, only 10 genotypes (71%) showed amplification for both MYMIV-MP (~900 bp) and MYMIV-NM (~1000 bp) gene regions (Fig. 2). For modified Gem-CTAB method, 11 (79%) and 12 (86%) genotypes exhibited amplification for MYMIV-MP and MYMIV-NM primers respectively (Fig. 2). Failure of virus specific primer amplification in certain genotypes could be due to insufficient amount of viral DNA in the extracted DNA samples. Inconsistent detection of virus from yellow mosaic affected legumes has been reported due to polyphenols and very low concentration of virus (Rouhibakhsh *et al*., 2008). No amplification was observed with DNA extracted from negative control plants devoid of begomovirus infection. Modified Gem-CTAB method evolved in the present study, basically differed from other methods in (i) reducing tissue sample and extraction buffer by 5 times (20 mg :200 µl) and processed in 0.5ml eppendorf tubes, (ii) incubation condition for lysis was carried out at 95˚C for 10 mins in a thermal cycler, (iii) DNA precipitation step was carried out with isopropanol and incubation at -80˚C for10 mins.

In RNA extraction, kit protocol followed by LiCl precipitation alone yielded satisfactory results for leaves, while kit protocol with all its modifications worked well for flowers and roots. Evaluation of total RNA extracted by different methods for various parameters is presented in Table 2.

Absorbance ratios for RNA extracted from kit+ LiCl precipitation method (A260/280 = 2.12 and A260/230 = 2.67) were more than that for all other protocols and indicative of pure RNA free of proteins and carbohydrates. Furthermore, RNA yield was enhanced from 0.1 µg/µl to 0.5 µg/µl when LiCl precipitation was carried out at -80˚C for 10 min. The quantity and quality of total RNA from flower and root tissues were found to be similar. Absorbance ratios, A260/280 and 260/230 for flowers and roots RNA extracted through different methods were shown in Table 2. In this study, use of double the amount of β-mercaptoethanol to kit lysis buffer, double washing of bound RNA on column and Tri-reagent were found to be ineffective in the removal of polysaccharides and polyphenolics contaminations from the blackgram leaf tissue. However, β-mercaptoethanol has been reported to prevents production of quinones from phenolics in bark tissues of *Hevea brasiliens* (Venkatachalam,1999). Poor quality of leaf RNA isolated by TRIzol followed by LiCl precipitation could be associated with carryover of contaminants in RNA pellet after LiCl precipitation and ethanol washing steps. This could also be because of co-precipitation of polysaccharides and secondary metabolites with RNA owing to similar physicochemical properties (Sharma *et al*., 2003; Key *et al*., 2012) that interferes in further downstream processing.

Total RNA from leaves isolated through kit protocol followed by LiCl precipitation was good in quantity and quality as evidenced from successful PCR amplification of putative gene PV18S (Fig. 3). Gene amplification was not detected when the RNA was isolated from kit method alone. This could be because of incomplete removal of secondary metabolites during kit method, however complete elimination of contaminants could be achieved only after LiCl precipitation of eluted RNA. LiCl precipitation do not efficiently precipitate DNA, proteins and carbohydrates (Barlow *et al*.,1963), remove inhibitors of cDNA synthesis from RNA preparations (Cathala *et al*.,1983) and washing with ethylalcohol (70-80%) effectively eliminated the salt residues present in the RNA pellet (Hidayah, 2013). Column based kit method for RNA isolation basically works efficiently by denaturing all cell components, immediate inactivation of RNases and efficient removal of non-nucleic acid based debris by facilitating binding of RNA to the silica membrane (Biswas and Ghosh, 2016). In addition, column based kits maximizes inactivation of DNA by co-precipitation during isolation process, by on column DNase digestion (Biswas and Ghosh, 2016). In case of flowers and roots, successful putative gene amplification from kit protocol and all three modifications incorporated in kit based method indicated effective removal of secondary metabolites from blackgram flower and root tissues. No putative *PV18S* gene amplification was observed for TRIzol and TRIzol followed by LiCl precipitation methods indicating same reason as assumed for leaf tissues. In this study, TRIzol was found to be ineffective in removal of secondary metabolites from all three different blackgram tissues: leaves, flowers and roots. Similar observations were reported for pigeon pea whole seedlings comprising of roots, shoot and leaves (Biswas and Ghosh, 2016). Moreover, in this study RNA isolated from leaves with TRIzol method was brown in colour and difficult to dissolve which may be attributed to co-precipitation of polysaccharides and oxidation of phenolic compounds that interact irreversibly with nucleic acids (Chan *et al*., 2004; Vasanthaiah *et al*., 2008). This suggests that the application of TRIzol requires additional purification protocols when used for legumes as seen for blackgram leaves (TRIzol + RNeasy Plant Mini Kit) (Kundu *et al*., 2015). Above observations suggests that kit protocol alone is effective for obtaining pure RNA from blackgram flowers and roots. It must be followed by LiCl precipitation to attain pure RNA from blackgram leaves. This is due to high concentration of phenolics and secondary metabolites in leaves compared to flowers and roots. Total RNA yield from leaves using kit followed by LiCl method was ~ 1 µg/µl (100 mg tissue) which is low compared to 36 µg/µl (100 mg tissue) or 360 µg/µl (1000mg tissue) from manual extraction method (Babu *et al*., 2014). However, quality is reported to be better compared to manual method as indicated from absorbance ratios (A260/A230).

In this study, modifications were incorporated in established Gem-CTAB method to make it fast and economical without compromising quality. This modified Gem-CTAB method may prove useful in extracting viral genomes from small lesion, extracting DNA from large number of samples and very less tissue sample. Similarly, for RNA isolation, Kit + LiCl precipitation method was found to be useful in isolating sufficient amount of good-quality RNA extraction from blackgram leaves rich in polysaccharides and phenolics.

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Table 1. List of primers used in this study.

Primer name	Sequence (5'–3')	Genome component	Size of anticipated products (bp)	References
Actin	GTTCTGTTCCAGCCATCCAT GTGGTGCGACAACCTTGATT	Blackgram genome	~200	Kundu *et al*., 2013; Maiti *et al*., 2012
MYMIV-NM	GTATTTGCAKCAWGTTCAAGA AGGDGTCATTAGCTTAGC	MYMIV	~1000	Naimuddin and Pratap, 2011
MYMIV-MP	ATGGAAAATTATTCAGGTGCA CTACAACGCTTTGTTCACATT	MYMIV	~900	Naimuddin and Pratap, 2011
Pv18S	TCGAAGCGATCTTTTCGTAGA TTCTCAGTCGACTCGCTTTTT	*Phaseolus vulgaris*	~250	Abid *et al*., 2011
YMV 83	TCACGAAGGTTACGACCAAA CGCATACTCGGTGTTATTGC	Blackgram cultivar TU94-2 contig 9788	~300	
YMV 93	CACCCTTCTGAGGGAAAACA CTCAAAGCATGGGTCAAGGT	Blackgram cultivar TU94-2 contig 2854	~150	
YMV 101	GGAATCAGAGGGCAAATTGA CGGATGCAATGTGAACAATC	Blackgram cultivar TU94-2 contig 318	~450	
YMV 102	TCCTCTCAACGGCCAATATC TATCACGCAGGCTTCCTTCT	Blackgram cultivar TU94-2 contig 3165	~350	
YMV 124	CATTAGCCCCACCACTTGTT GGGTCCTCTCACTGGATGAA	Blackgram cultivar TU94-2 contig 4768	~250	

Electronic Journal of Plant Breeding, 10 (1): 167 – 176 (Mar 2019) DOI: 10.5958/0975-928X.2019.00020.6
ISSN 0975-928X

Table 2. Evaluation of different DNA and RNA extraction methods for various quality parameters.

No.	Method	Tissue type	Quantity of tissue (mg) and Final volume (µl)	Yield (ng/µl)	Integrity on agarose gel	Absorbance Ratio A260 /280	A260 /230	Region amplified	No. of PCR positive samples /No. of samples tested
	DNA								
1	Gem-CTAB method	Leaf	100, 100	940	good	1.97	2.05	Actin	14/14
								MYMIV-MP	10/14
								MYMIV-NM	10/14
2	Modified Gem-CTAB method	Leaf	20, 20	3000	good	2.00	2.17	Actin	14/14
								MYMIV-MP	11/14
								MYMIV-NM	12/14
	RNA								
1	Spectrum™ Plant Total RNA Kit	Leaf	100, 50	958	Intact	1.95	1.92	PV 18S	No
		Flower		218	Smeared	2.13	0.86	PV 18S	Yes
		Root		236	Smeared	2.09	2.12	PV 18S	Yes
2	Spectrum™ Plant Total RNA Kit + 2 x mercaptoethanol	Leaf	100, 50	1023	Intact	1.99	1.97	PV 18S	No
		Flower		258	Smeared	2.14	1.31	PV 18S	Yes
		Root		260	Smeared	2.12	1.39	PV 18S	Yes
3	Spectrum™ Plant Total RNA Kit + 2 x on-column washing	Leaf	100, 50	1219	Intact	1.96	1.75	PV 18S	No
		Flower		259	Smeared	2.13	1.18	PV 18S	Yes
		Root		233	Smeared	2.01	0.92	PV 18S	Yes
4	Spectrum™ Plant Total RNA Kit + Licl2 precipitation + -80^{o} C (10 min)	Leaf	100, 50	465	Intact	2.16	2.67	PV 18S	Yes
		Flower		242	Smeared	2.13	2.27	PV 18S	Yes
		Root		285	Intact	2.15	2.26	PV 18S	Yes
5	Trizol reagent	Leaf	100, 50	1551	Intact	1.67	0.41	PV 18S	No
		Flower		789	Smeared	1.14	0.21	PV 18S	No
		Root		374	Smeared	1.83	0.77	PV 18S	No
6	Trizol reagent + Licl2 precipitation + -80^{o} C (10 min)	Leaf	100, 50	742	Intact	1.89	1.82	PV 18S	No
		Flower		20	Smeared	3.05	0.30	PV 18S	No
		Root		133	Smeared	1.88	1.58	PV 18S	No

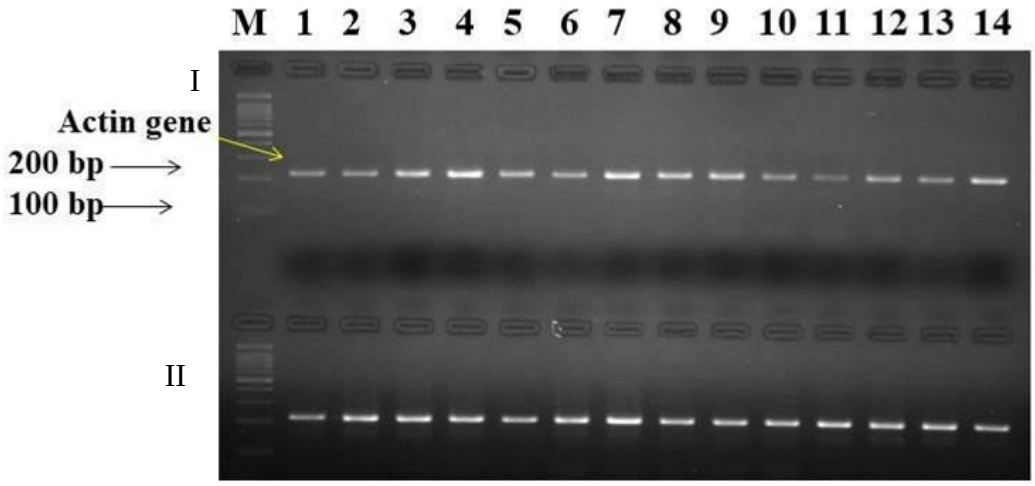


Fig. 1. **PCR amplification of *actin* gene from leaves of 14 blackgram genotypes.I: Gem-CTAB method and II: Modified Gem-CTAB method. M:100bp ladder.**

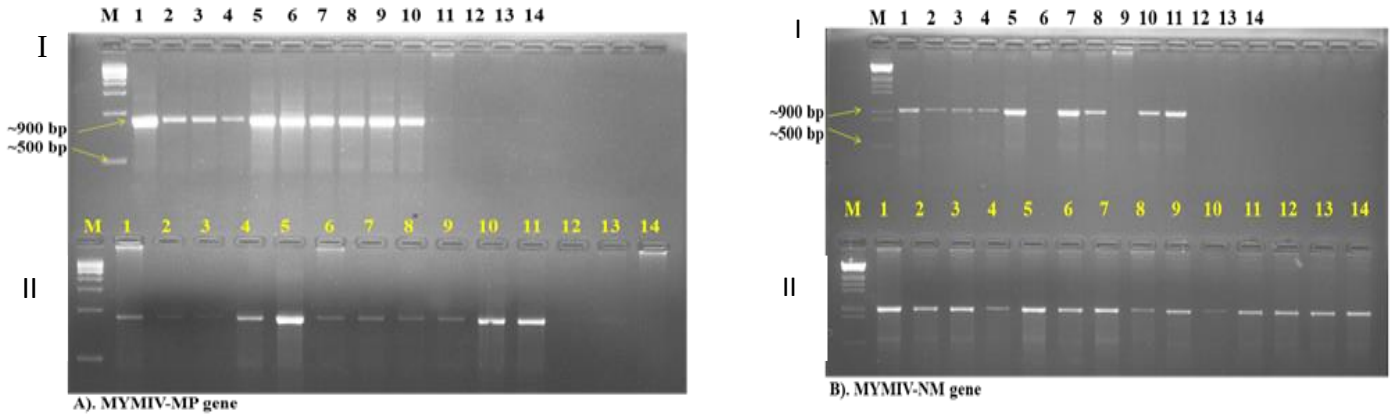


Fig. 2. PCR amplification of Begomovirus specific gene fragment A)MYMIV-MP and B) MYMIV-NM from leaves of 14 blackgram genotypes. I: Gem-CTAB method and II: Modified Gem-CTAB method. M:500bp ladder.

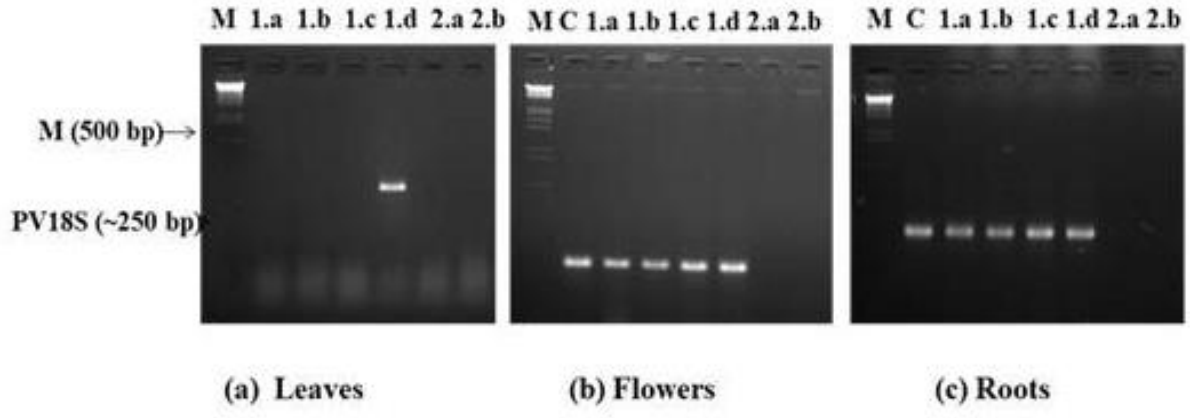
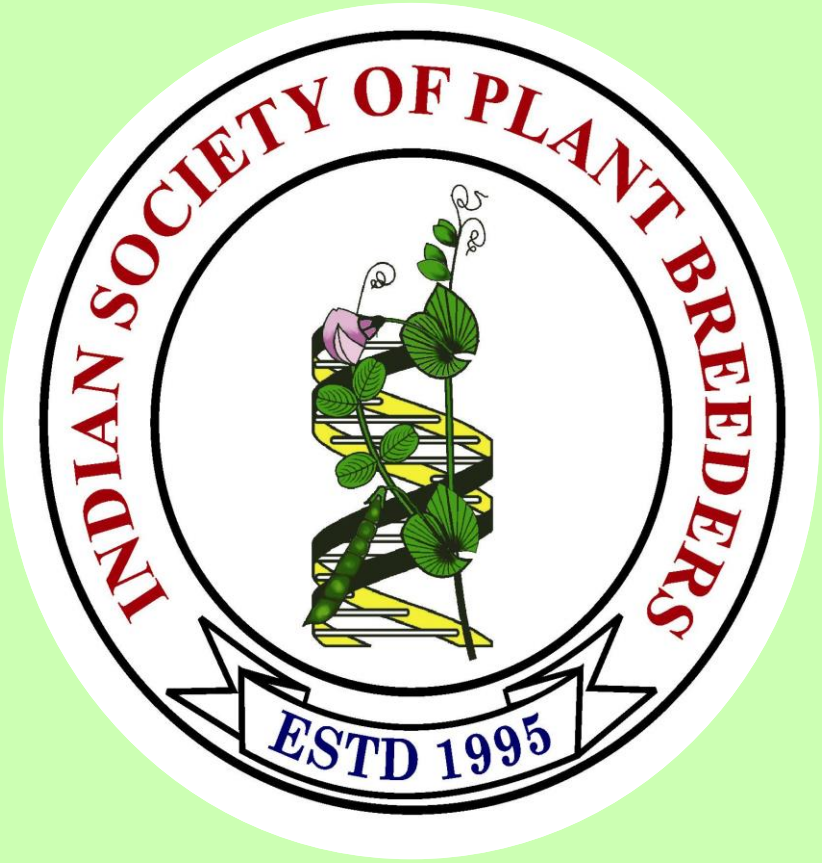


Fig. 3. PCR amplification of *Phaseolus vulgaris* 18 S RNA gene fragment from cDNA (a) leaves, (b) flower and (c) root tissues of blackgram.(d): Genomic DNA from blackgram seedling, 1.a) column based kit,1.b) kit with twice amount of mercaptoethanol in lysis buffer, 1.c) kit with twice on-column washing , 1.d) kit followed by LiCl precipitation, 2.a) TRIzol and 2.b)TRIzol followed by LiCl precipitation. M: Molecular marker 500 bp.



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