



Optimization of an Efficient RNA Isolation Protocol from *Fusarium* wilted Pigeonpea Plant (*Cajanus cajan* (L.) Millspaugh)

Koushik Biswas^{1*} and Parthadeb Ghosh²

¹Department of Biotechnology, Shri Jagdish Prasad Jhabarmal Tibrewala University, Vidyanagri, Jhunjhunu, Churu, Rajasthan, India.

²Department of Botany, University of Kalyani, Kalyani, Nadia, West Bengal, India.

Authors' contributions

This work was carried out in collaboration between both authors. Author PG designed the study and supervised the work. Author KB carried out all laboratories work, managed the analysis of the study and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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Method Article

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ABSTRACT

Aims: The present study demonstrated a comparative evaluation of RNA isolation from pigeonpea by using conventional or modified laboratory based methods as well as optimizing the existing well known protocol to extract a satisfactory amount of purified and reproducible RNA required for downstream processing.

Study Design: Experimental.

Place and Duration of Study: Plant Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Kalyani, Kalyani, Nadia, West Bengal, India in 2015.

Methodology: In the present investigation, an attempt was made to optimize the method for getting high quality RNA from healthy pigeonpea seedling as well as inoculated with fungal strain of *Fusarium udum* (FU) Butler. After optimizing the method showing best performance among all

*Corresponding author: E-mail: koushik_hp@rediffmail.com;

experimenting protocols, the RNAs were further validated for quality by reverse transcription polymerase chain reaction (RT-PCR) for amplification of plant specific Ascorbate peroxidase gene (APX) and FU specific Cellobiohydrolases (CBHs) gene responsible for infectivity of *Fusarium udum*.

Results: The determinant applications of Guanidium thiocyanate (GITC) and sodium citrate based lysis buffer for efficient extraction at initial step and an effective precipitation by using isopropanol and sodium chloride in the final step made a successful optimization of the excellent quality of total RNA isolation. The same RNA was self sufficient to identify the traces of plant and fungal specific gene through RT-PCR.

Conclusion: This observation under certain modifications could be effective to get RNA of excellent quality with the elevated yield from extremely challenging high polysaccharides and polyphenolic rich pigeonpea plant. Simultaneously this experimental design will not only provide better options for the identification of rare transcripts involved in resistance mechanisms in pigeonpea against various stress responses, but also make an opportunity to study the plant pathogen interaction at the molecular level.

Keywords: RNA; pigeon pea; Fusarium udum; reverse transcriptase – polymerase chain reaction.

1. INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh], a summer-season legume cultivated throughout tropical and subcontinent of Asia and Africa, is an essential source of rich dietary protein for human consumption. Though the nutritional value of this crop is much more significant for the world vegetarian population, unfortunately its productivity is not satisfactory in most of the pigeonpea growing areas making a significant gap between the attainable yield and the experimental yield potential. The major constraint in pigeonpea production is considered to be biotic stresses, which cause 30-100% yield losses in susceptible genotypes [1]. Therefore, knowledge of understanding the genetic basis for multiple stress tolerance is a vital factor in discovering the way out for genetic improvement of pigeonpea. There is an urgent need to improve the functional genomics, transcriptomics of pigeonpea for identifying novel genes for biotic stress tolerance.

Legume crops including pigeonpea are rich in polysaccharides, starch, polyphenolics and other secondary metabolites which make the nucleic acid isolation quite difficult, as they tend to degrade and co-precipitate with the nucleic acid especially RNA. It is also well established that there is a higher tendency of accelerating the concentration of these compounds when the plants are exposed to various biotic and abiotic stresses, such as pathogen infection or drought stress [2-4]. Because of sensitivity to oxygen, all the polyphenolic components become oxidized to quinones making the nucleic acids indivisible due to their covalent bonding [5]. Other

troubleshooting components are polysaccharides and secondary metabolites that highly co-precipitate with RNA due to having similar physicochemical properties like RNA [6,7].

There are a number of improved and modified methods reported for isolation of RNA from leguminous plants *viz.* modified Guanidine thiocyanate method for polysaccharides, polyphenolics and nucleases rich plants [8], Urea-Lithium chloride (LiCl) lysis buffer based protocol for Urdbean, Horsegram and Pigeonpea [9], Sodium chloride (NaCl) and Tris-Hydrochloride (Tris-HCl) based extraction buffer protocol for Blackgram [10], modified Borax decahydrate extraction buffer based method for lentil [11] and in some non-leguminous plants, Guanidine hydrochloride with sarcosyl based buffer for wheat [12], Cetyltrimethylammonium bromide-based protocol for black berry [13], combination of 3% Cetyl trimethylammonium bromide (CTAB) and Sodium dodecyl sulfate (SDS) based extraction buffer method for straw berry [14] and combination of CTAB and silica column based commercial plant RNA extraction kit (RNeasy® Plant Mini Kit, Qiagen, Germany) for *Jatropha* [15]. But no one of the above methods has been optimized for RNA isolation from either healthy or fungal stress imposed pigeonpea seedlings, which could precipitate enough quantity of good quality RNA favorable for high throughput transcript profiling studies. In this study, we aimed to standardize a best suited protocol for RNA isolation from *Fusarium udum* (FU) wilted pigeonpea seedlings and compared it with some improved methods reported earlier like Trizole based single step isolation method (Ambion, USA), Urea-LiCl lysis buffer based protocol [9],

Sodium acetate (NaOAc) based extraction buffer method and even highly appreciable silica column based protocol (Macherey-Nagel, Germany). The identified best protocol for isolation of RNA was then improved by amendment in some steps to get highly reproducible and pure RNA. The purity of isolated RNA was further checked and used in downstream application by performing RT-PCR for amplifying pigeonpea plant specific Ascorbate peroxidase gene (APX) and FU specific Cellobiohydrolases (CBHs) gene responsible for disease development.

2. MATERIALS AND METHODS

2.1 Plant Materials and Biotic Stress Treatment

Apparently healthy seeds of Fusarium wilt susceptible variety (ICP2376) of pigeonpea were surface sterilized with 2% Sodium hypochlorite (NaOCl) for 3 minutes followed by sterile water for three times. Five to six seeds were sown in polythene bags filled with sterilized river sand and kept for 6-7 days in greenhouse maintained at $25\pm 2^{\circ}\text{C}$.

Seven days old pigeonpea seedlings were uprooted and inoculated with a virulent strain of *Fusarium udum*. Standard root-dip inoculation method was implied using conidial suspension of 6×10^5 spores/ml. Thereafter inoculated seedlings were transplanted into the pots and kept in the greenhouse ($25\pm 2^{\circ}\text{C}$). After 7 days of inoculation, the seedlings were uprooted from the soil and washed with sterile water and immediately blot dry using sterile filter paper (Fig. 1). After drying the harvested samples, they were immediately snapped frozen in liquid nitrogen and stored at -80°C until further use [16].

2.2 Identification of the Paramount Protocol of RNA Isolation from Pigeonpea

For performing all protocols, usual precautionary measures were followed as: the chemicals used were of molecular biology grade and all solutions were made by using nuclease free water. Consumables were of Ribonuclease (RNase) free; pipette tips and plastic-wares were treated overnight with 0.1% Diethylpyrocarbonate (DEPC) treated distilled water, followed by autoclaving and complete drying to remove any traces of DEPC. Mortars and pestles were also

autoclaved after DEPC treatment. Five standard RNA isolation methods were performed to identify best suited paramount protocol of RNA isolation from pigeonpea and these can be described in the following way.

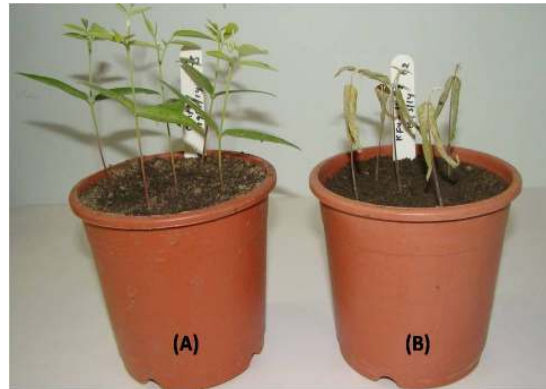


Fig. 1. Plant materials (Whole seedling) collected from healthy (A) and Fusarium wilted (B) Pigeonpea cv. ICP2376 after 7 days of FU inoculation

2.2.1 Urea-LiCl lysis buffer based protocol

This protocol is specially designed earlier for RNA isolation from seeds of three leguminous plants which were *Vigna mungo*, *Dolichos biflorus* and *Cajanus cajan* [9]. The lysis buffer used in that case was Urea (8M) and LiCl (4M) where as resuspension buffer comprised of NaCl (0.1M), SDS (2%), Tris (0.01M, pH 7.5), Polyvinylpyrrolidone (PVP) at 2.5% and the chelating agent Ethylenediaminetetraacetic acid (EDTA) (25mM). In addition, the lysate was passed through the QIAshredder column (Qiagen, Germany) for total removal of cell debris. For effective precipitation of total RNA, 3M of sodium acetate was separately added to isopropanol and kept at -20°C for 2 hrs. The purified RNA was experimented further for quality in 1% agarose gel.

2.2.2 Trizole based single step isolation method

TRIzol Reagent is a ready-to-use reagent and designed to isolate high quality total RNA from all types of cells and tissues. Here we used 1ml of TRIzol® reagent (Ambion, USA) for extracting and making lysate from 50 mg of whole crushed pigeonpea seedlings. In general all the recommended materials such as chloroform, isopropanol and 75% ethanol are strictly used as

per user guideline. Finally, RNA pellet was dissolved in 50 μ l nuclease free water for further quality checking and other downstream experiments [17].

2.2.3 Silica column and kit based method

We have selected a standard total RNA isolation kit commercially named as NucleoSpin® RNA Plant kit (Macherey-Nagel, Germany) which ensures on column removal of contaminating DNA at final steps. The bounded RNA was finally eluted by applying 50 μ l of nuclease free water at low ionic strength and further experimented for RNA quality in 1% agarose gel.

2.2.4 Sodium acetate based extraction buffer method

A modified NaOAc based lysis buffer was prepared and used for the extraction of RNA from all the tissues of pigeonpea. The whole tissues of FU infected pigeonpea seedlings were finely powdered by grinding in liquid nitrogen and 50 mg of fine powder from two sets of samples were used as starting material for all five protocols mentioned here. The lysis buffer contained 1.5 M NaOAc, 75 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 7.5) and 2% 2-Mercaptoethanol. 100 mg of ground tissue was added to 1 ml of lysis buffer in 2 ml microfuge tube and 100 μ g of proteinase K was added separately. The mixture was then vortexed vigorously for 10 sec and incubated at 55°C for 20 min for tissue lysis. The samples were incubated on ice for 5 min followed by centrifugation at 5,000 revolutions per minute (rpm) for 5 min. Without disturbing the debris, the supernatant was then transferred into a fresh microfuge tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added per sample and subsequently centrifuged at 12,000 g for 10 min at 4°C. The upper aqueous phase was transferred carefully into a new microfuge tube and precipitation of total nucleic acid (Deoxyribonucleic acid-DNA and RNA) was carried out in the presence of one fourth volume of 1.5 M NaOAc and equal volume of isopropanol by keeping in low temperature (-20°C) for 2 h. After precipitation the solution was centrifuged at 13000 rpm for 20 min at 4°C. After decanting the whole supernatant from the tube, the pellet was washed with 75% pre-chilled ethanol for two times by centrifuging at 13000 rpm for 5 min at 4°C. The final pellet thus obtained by removing the excess ethanol was air dried at 37°C for 15-20 min in the incubator. The RNA pellet dried at the bottom of the tube having RNA pellet was

re-suspended in 50 μ L RNase-free water. To eliminate genomic DNA contamination, 5 μ L Deoxyribonuclease-I (DNase I) (1U/ μ L) was added to the solution and incubated at 37°C for 30 min and the treated RNA was kept in -80°C until further use.

2.2.5 Modification of suited protocol for optimizing RNA isolation

After getting a better result by following GITC based protocol [18] in comparison to other methods mentioned above, we further have modified this extraction method in some steps to achieve an impressive quality and adequate quantity of RNA. Before starting of RNA isolation by GITC based extraction method, the modified extraction buffer "Solution D" was prepared separately as per requirement described in Table 1. 100 mg of finely ground tissue powder of pigeonpea was transferred to an oakridge tube containing 5 ml of Solution D where 2-Mercaptoethanol and Sodium lauroyl sarcosinate were separately added as per concentration recommended. The mixture was then vigorously vortexed and homogenized for 20-25 sec at room temperature. There after the homogenate was centrifuged at 9000 rpm for 20 min at 4°C followed by transferring the upper aqueous phase carefully to a fresh tube. An equal volume of chilled isopropanol and one fourth volume of 3M NaOAc solution (pH 5.2) were added to the aqueous sample and mixed well. The mixture was kept in -80°C for 2 hrs to allow precipitation and the precipitated nucleic acid was collected by centrifuging at 12000 rpm for 30 min at 4°C. The pellet was collected by carefully decanting the isopropanol and re-suspended in 0.3 ml of solution-D per 1 ml of extraction buffer initially used. Again 1 ml of chilled isopropanol and referred amount of NaOAc solution were added to the mixture and allowed to precipitate at -80°C for 1 hrs after vortexing thus making the precipitation more strong. The precipitated nucleic acid was collected in a new microfuge tube by spinning at maximum speed for 10 min at 4°C. After that the nucleic acid pellet was air-dried and re-suspended in 1M NaCl with a volume of 1 ml. The entire solution was transferred to 2 ml microfuge tube and kept on ice for 5 min followed by centrifuging at maximum speed for 10 min at 4°C. After removing the entire salts from the tube, the pellet was washed with 75% ethanol by centrifuging the tube at 10000 rpm for 5 min at 4°C. The pellet was air dried shortly and re-suspended in 50 μ L RNase-free water. The DNase treatment of the

isolated RNA was performed as described in fourth protocol.

2.3 Quantification and Quality Checking of Total RNA

For all the methods used for RNA isolation in this study, the quantity and quality were measured spectrophotometrically at 230, 260 and 280 nm where protein and DNA contamination was assessed from the A260/230 and A260/280 ratio appeared in spectrophotometric reading. Quality and integrity of RNA were also verified by electrophoresing all the isolated RNA on 1.5% agarose gel followed by staining with ethidium bromide [18]. All the bands were checked and photographed using Gel documentation unit.

2.4 RT-PCR Based Detection of Plant and Fungal Genes for Downstream Application

Total RNA isolated from FU inoculated and healthy seedlings of pigeonpea by using the modified protocol 5 (GTC based extraction method) was reverse transcribed using Oligo-(dT) primer from 1µg of template RNA by reverse transcriptase enzyme. First-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA). To carry out PCR reaction,

specific oligonucleotide primers (Table 2) designed by retrieving gene sequences available in NCBI database (Pigeonpea specific Ascorbate peroxidase gene and FU specific Cellobiohydrolases gene) by the online IDT tool (Integrated DNA Technology). To identify the plant specific APX gene and fungal CBHs gene, PCR was performed in separate microfuge tubes consisting of total volume of 25 µl reaction mixture with the following components: designed primers (10 mM, 0.5 µl each), dNTPs of (25 mM) 0.25 µl, MgCl₂-1.25 µl, 2.5 µl of DNA polymerase buffer (10×) and 1U of Taq Polymerase (Merck, USA) along with 2 µl of synthesized cDNA. Amplification was achieved in an automated thermal-cycler (GeneAmp PCR9700 system, Applied Biosystems, USA) by optimizing the reaction conditions for APX and CBHs gene individually. For APX gene, the optimized conditions are as follows: Initial denaturation (94°C for 3 min.) followed by 35 cycles composed of denaturation (95°C for 45 sec), annealing temperature (60°C for 45 sec) and extension (72°C for 30 sec) followed by a final extension of 5 min at 72°C temperature. In case of CBHs gene, all conditions kept remain same where the annealing was optimized at 56°C for 30 sec. The PCR products were analyzed on 1.0% agarose/EtBr gel to check the desirable amplification of DNA bands for both of the plant and fungus specific genes.

Table 1. Details of components used to prepare extraction buffer 'Solution D' in the modified method of GITC based RNA isolation protocol

Sl. no.	Components	Concentration
1	Guanidium thiocyanate (GITC)	4 molar
2	Sodium Citrate (pH-7.0)	25 milimolar
3	Sodium chloride	1.5 molar
4	2-Mercaptoethanol	0.1%
5	Sodium lauroyl sarcosinate	0.5%

Table 2. List of primers pairs used for RT-PCR based plant and fungal gene identification in this study

Sl. no.	Gene specific primers	Primer sequence	Annealing temperature
1.	APX	For: 5'ACGGTCTCGATATCGCTGTTAG 3' Rev: 5'GTGAGTTGTCAAATATAAGAGGGTCTG3'	60°C
2.	CBHs	For: 5'AGTCAGTATGCGATGTTTCTGT3' Rev: 5'CAACGGACGCTTTCTGAGAT3'	56°C

3. RESULTS AND DISCUSSION

3.1 RNA Isolation Standardization

In the present study, we focused to modify some predominantly well established RNA extraction protocols which were mainly based on Trizole reagent and guanidium thiocyanate (GITC) extraction method [18], commercial RNA extraction kit (Macherey-Nagel, Germany), NaOAc based extraction buffer method and as well as Urea-LiCl lysis buffer based protocol [9] specifically designed for RNA isolation from leguminous seeds of healthy and FU infected whole seedlings of pigeonpea. But a high yield and quality of total RNA was only achieved after

some critical modifications of the GITC based extraction method reported in this study (Fig. 2, Table 3). It was observed that all well established protocols used in this study did not yield satisfactory results with respect to high yield and good quality pigeon pea RNA for efficient use in further downstream experiments. Furthermore, the improved method with some modifications of GITC based RNA extraction protocol were used to isolate RNA from high amount of polysaccharides and polyphenolic rich whole pigeonpea seedlings (contain sufficient quantities of roots, shoots and leaves) and this significantly gave an excellent result with good RNA quality (ratio 260/280 2.00 ± 0.2) (Fig. 2f) and elevated yield ($1.1 \pm 0.2 \mu\text{g RNA}/\mu\text{l}$ of sample).

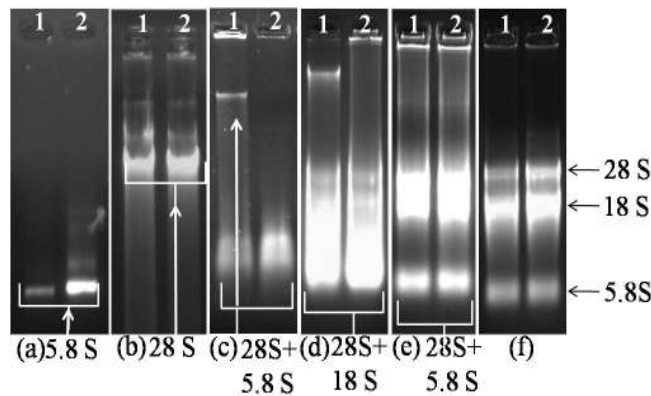


Fig. 2. Comparative study of different RNA isolation methods from pigeonpea along with the visible RNA of variable sized fractions (Svedberg unit) where lane 1 indicates RNA from control plant and lane 2 indicates for RNA from FU infected plant

(a) Trizole three layer isolation method (Ambion, USA). (b) Urea-LiCl lysis buffer based protocol (Kansal et al., 2008). (c) NucleoSpin® RNA Plant kit-RNA isolation from plant (Macherey-Nagel, Germany). (d) Sodium acetate based extraction buffer method. (e) Guanidium thiocyanate based extraction method (Sambrook and Russell, 2001). (f) Improved version of Guanidium thiocyanate based extraction method

Table 3. Total RNA yields isolated from pigeonpea seedling samples by using five different protocols with some modifications. (a)-Healthy sample, (b)-FU infected sample

Isolation method	RNA yield (ng/ μl)	RNA purity	
		A260/A280	A260/A230
Protocol-1 (a)	390	1.94	1.97
Protocol-1 (b)	407	1.92	1.97
Protocol-2 (a)	142	1.76	1.89
Protocol-2 (b)	334	1.68	1.88
Protocol-3 (a)	384	1.93	1.98
Protocol-3 (b)	361	1.89	1.95
Protocol-4 (a)	1632	1.78	1.81
Protocol-4 (b)	1553	1.70	1.76
Protocol-5 (a)	1595	2.10	1.98
Protocol-5 (b)	1478	2.04	1.94
Protocol-5 (a) [Improved method]	1124	2.10	2.00
Protocol-5 (b) [Improved method]	1194	2.02	1.93

The first method (Urea-LiCl lysis buffer based protocol) was established earlier [9] and effective for high quality RNA isolation from the seeds of Pigeonpea, Mustard and Spinach like leguminous crops. In that study, the isolated RNA from pigeonpea seeds were reported with a concentration less than 0.5 µg per µl of sample (Yield ~5.4 µg in 15 µl RNA) and that could be useful for normal molecular analysis but could not allow high throughput molecular biology experiments viz. Subtractive Hybridization, Oligonucleotide microarray and Whole transcriptome sequencing etc. The improved GITC method employed in the present study contributed a higher yield, more than 1.0 µg per µl of RNA sample (Table 1), with reproducible quality (Fig. 2f) which is more adequate to perform high throughput RNA based techniques [19]. The same protocol was used here to extract RNA from pigeonpea seedlings which typically showing band separation with a clear band of 28S ribosomal RNA (rRNA) but an overall degradation of all types of rRNA throughout the lane (Fig. 2b). By following protocol-1, the lower quality of obtaining RNA could be associated with selective but weak precipitation of RNA by lithium chloride (4M) and ineffective removal of polysaccharides and polyphenolics contaminations from the tissues of whole seedling by resuspension buffer containing 2.5 % of Polyvinylpyrrolidone (PVP). In contrast to the activities of these chemicals, crude Guanidium salts effectively disrupted all types of cells followed by solubilizing their components and most importantly denatured any traces of RNases from the sample which could be considered as the most important negative factor to degrade the extracted RNA from pigeonpea tissues by implying most of the protocols here [18]. Another important point is the selective and stable precipitation of nucleic acid which can be optimized by several salts but the best result in efficient precipitation was finally achieved by using 1M NaCl in GITC based protocol under the study and which was earlier established for efficient plant viral DNA isolation [20].

The second protocol for RNA isolation performed in the current study was Trizole based single step isolation method (Ambion, USA). The RNA obtained after extraction was low in quantity with a spectrophotometric concentration of 0.33 µg per µl of sample but poor quality showing A260/230 ratio (Table 1). As far as rRNA bands are concerned, the single and prominent band appeared in both control and FU infected RNA samples is of 5.8S (Fig. 2a) where band

separation is slightly observable in lane 2 but difficult to distinguish 28S and 18S rRNA because of their ample appearance in gel (Fig. 2a). This result confirms that trizol reagent played here a better role in comparison to protocol 1 [9] by maintaining the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization [17]. But simultaneously it was unable to precipitate specific large size of rRNA (28S and 18S) due to the presence of less quantity of GITC which it is capable enough to separate total RNA actively but only when present in sufficient amount. Therefore the isolated RNA is not suitable for detecting promising plant genes and disease causing fungal genes (applicable for FU infected samples) by RT-PCR based molecular analysis.

Column and commercial kit based RNA isolation basically works efficiently by denaturing all cell components and immediate inactivation of RNases with the help of one type of chaotropic ions (GITC) present in the initial lysis buffer where the efficient removal of non-nucleic acid based debris is achieved by creating appropriate binding conditions which favor adsorption of RNA to the silica membrane. Another advantage of using commercial column based kits is maximum inactivation of DNA, which co precipitates during isolation process, by on column DNase digestion. In the present study, we have picked up a high throughput RNA isolation kit (NucleoSpin® RNA Plant kit, Macherey-Nagel, Germany) as a standard column based commercial method which ensure the yield of RNA with better integrity and high quality than the TRI reagent based protocol [21]. But the kit based method failed to isolate good quality with sufficient quantity of total RNA from FU infected and healthy pigeonpea plants where the isolated RNA shows an overall degradation of all rRNA by representing a smear band of high molecular weight (Fig. 2c). These inferior results could be based on the roles of chemicals used in the kit protocol. The presence of GITC in lysis buffer was not self sufficient in quantity to disrupt all types of cells associated with polysaccharides and polyphenolics content in pigeonpea. More over the kit based method does not offer the flexibility for different plant cell types that need to be considered for some specific conditions. In another way, the present improved method complementing the adequate use of crude GITC that does not jump over the cost of the more expensive kits is generally used commercially. The final concentration of the strong denaturant,

GITC was optimized upto 4 M for pigeonpea tissue that efficiently inhibits the RNase activity to let the nucleic acids easily available in upper aqueous phase of the solution using centrifugal force [18].

The fourth most common protocol is basically designed to extract nucleic acids followed by selective isolation of RNA with the help of NaOAc based extraction buffer. This salt is readily available and widely used for selective and efficient precipitation of nucleic acid in combination with isopropanol or ethanol. Earlier NaOAc was used to extract high quality total RNA from polysaccharides and polyphenolics rich plant tissues [8], crop seeds like wheat, barley and maize [12], Horse gram, black gram and pigeonpea seeds [9], *Melastoma decemfidum* like shrub [22] and from *Reaumuria soongorica*, a Desert Plant [23]. The salt in current protocol was used two times, but initially it displayed a depressive action in lysis buffer rather than GITC used in modified protocol. Thus GITC was proved to be more efficient to release and separate the nucleic acid from crude sample and make the RNA intact (Fig. 2f) by inhibiting the RNase activity as compared to NaOAc salt (Fig. 2d). Another most probable reason for inferior recovery of nucleic acid is the use of 2-Mercaptoethanol and Proteinase K that partially felt to remove the protein and other organic parts from the sample as Sodium Lauroyl Sarcosinate. In other hand this component is not expected to be potentially toxic or harmful like SDS and other anionic detergents used in RNA isolation.

The GITC based method was followed directly for pigeonpea RNA isolation shown better yield

response ranging from 1.4-1.6 µg per µL RNA sample (Table 1) with a clear separation of high molecular weight (28S and 18S) and low molecular weight (5.8S and 5S) fraction of RNA bands (Fig. 2e). But the isolated RNA did not confirm the its intact form where there is no further separation observed among high molecular weight RNA of 28S and 18S fraction and also contaminated with enough quantity of DNA still after DNase treatment. Improved version of GITC method has overcome both the mentioned problems where repeated addition of 3M NaOAc solution with isopropanol has minimized the chances of co-precipitation of DNA and residual polysaccharides from the RNA sample in a short period to obtain an enhanced quality of intact RNA. Further suspension of nucleic acid pellet with 1M NaCl has removed any traces of polyphenolics and polysaccharides by complete solubilizing into the aqueous phase of salt solution. Thus the improved GITC method ensured the excellent quality of integral RNA with sufficient quantity which can be further satisfy to conduct any type of high throughput molecular experiment at advanced level.

3.2 Utility of Isolated RNA for Both Fungal and Plant Downstream Experiment

The total RNA isolated by the improved method from healthy and FU infected pigeonpea was further utilized in reverse transcription to confirm its applicability in efficient detection of plant and fungal candidate genes. The plant candidate gene selected here to design primers in RT-PCR was APX, well known for its induction activity under multiple stress conditions by destroying the increasing level of harmful reactive oxygen

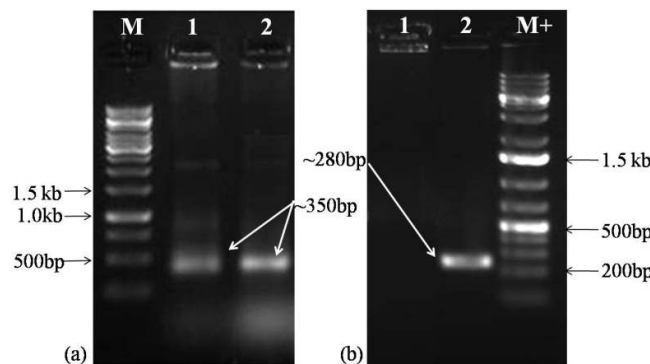


Fig. 3. Downstream application of the modified method derived high quality RNA by RT-PCR method

(a) PCR based validation of plant specific APX. (b) PCR based validation of FU specific CBHs gene. M-1kb DNA ladder, M+-1kb+ DNA ladder, 1-Control sample, 2-FU infected sample

species especially Hydrogen peroxide (H₂O₂) [24]. In relation to the attack of FU, Cellobiohydrolase gene can be considered as a potent cellular enzyme secreted by fungi to degrade host plant cellulose through the discharge of cellobiose for the deployment of their pathogenic activities [25]. Therefore, this gene is extremely used as a target for identification of different *Fusarium* species in diseased plants [26]. The putative plant gene was successfully amplified with an expected amplicon size of 350 bp from both healthy and FU inoculated RNA preparations demonstrating the effective detection of plant candidate genes displaying better intensity in FU stressed sample (Fig 3a). The abundance of fungal gene Cellobiohydrolase is confirmed only in FU infected sample with a desirable size of 280bp where no amplification confirmed the absence of mRNA transcripts in total RNA isolated from the control sample (Fig 3b). This study makes an opening for major high throughput transcript profiling techniques like RNA sequencing, qPCR, Subtractive library construction, microarray and whole transcriptome sequencing techniques where starting RNA quality and quantity is prime the factor for successful finishing.

4. CONCLUSION

In conclusion, our modified method for RNA isolation proposes a high-yield and good-quality extraction of total RNA from highly mucilage and phenolics contains tissues of *C. cajan*. The modified protocol is suitable for sufficient amount of good-quality RNA extraction from *C. cajan* that contains high mucilage and phenolics where overall RNA quality is extremely dependent on the presence of several secondary metabolites. Moreover, this method is equally useful to extract plant RNA from biologically stressed samples as well as fungal RNA from the same source with maximum reproducibility to trace their gene's functions and behavior under various stresses occurred during pathogen attack.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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