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AN IMPROVED AND OPTIMIZED METHOD OF HIGH QUALITY RNA ISOLATION FROM RECALCITRANT PLANT *Ziziphus mauritiana* LAM FOR FUNCTIONAL GENOMIC STUDIES.

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ABSTRACT

Isolation of high quality of RNA is prerequisite for functional genomics studies. It is quite difficult to get quality RNA from recalcitrant tissues, due to large amounts of secondary metabolites and other interfering compounds. We have encountered this problem in isolation of RNA from the *Ziziphus mauritiana* Lam which is a xerophytic plant. To overcome this problem, we have developed an improved CTAB-PVPP-LiCl method. In this protocol we have used insoluble PVPP for removal of polysaccharides and phenolic compounds and acidic phenol for efficient partition of RNA. In addition to these two modifications, 8M LiCl precipitation step was included for preferential precipitation of RNA. The RNA isolated was found to be of high quality from both roots and leaves of normal and abiotic stressed plants as evidenced from the downstream applications such as RT-PCR and qRT-PCR.

KEYWORDS: ***Ziziphus mauritiana*, Polysaccharides, Polyphenols, RNA isolation, RT-PCR, qRT-PCR**

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INTRODUCTION

Ziziphus mauritiana Lam, belongs to family Rhamnaceae, commonly known as Indian Jujube or ber is mainly used as food, fodder, nutrient, medicine, construction material and fuel[1].It is an important medicinal plant consisting of varied secondary metabolites such as alkaloids, flavonoids, terpinoids, saponins and lipids. Moreover, ber is an extremely tolerant to biotic and abiotic stress and a dominant component of the natural vegetation in the desert[1, 2]. The wide range of potential uses of this plant is one of the reasons for the growing attention it is receiving as choice of fruit crop for problematic soil. However, molecular investigations on this plant are inadequate. To understand molecular mechanisms such as signal transduction, gene regulation and gene expression; an array of techniques such as reverse transcription polymerase chain reaction (RT-PCR), RACE, Northern hybridization, cDNA library construction and microarray analysis are very much essential[3] .Isolation of pure and un-degraded RNA is the key requisite for all these methods. In the literature many specific protocols appropriate for plants have been developed and extensively modified[4,5,6,7,8] and a choice of commercial kits supplied by the vendors are also available for the isolation of RNA from a variety of tissues. However, most of these methods and kits are not suitable for the isolation of good quality of RNA from perennial woody plants which are rich in secondary metabolites[9]. In our studies we have identified that *Z.mauritiana* as heavy metal tolerant plant (unpublished data). To understand the molecular mechanisms of heavy metal tolerance of this plant as a first step we tried to isolate total RNA from the roots and leaves of *Z.mauritiana* with a standard guanidinium-salt based method [10]; modified guanidinium method[11] and phenol/SDS based methods[12] and also the commercial vendor kits such as TRIzol reagent (Ambion, USA), NucleoSpin® RNA Plant (Macherey-Nagel, Germany). All these methods failed to yield good quality RNA from leaves and roots of *Z.mauritiana*. This could be because of the guanidinium salts present in the extraction buffers that might have interfered with the high amount of secondary metabolites which are present in this plant. RNA might be lost in the complex due to the ineffectiveness of the guanidinium salt in dissociating RNA from non-protein complexes during the isolation procedure [13, 14]. In the literature there exist alternative methods which are free of guanidinium salt for the isolation of good quality of RNA from the tissues containing high levels of phenolic compounds or carbohydrates[15, 6]. Nevertheless, not all of these methods have been successful in isolating high-quality RNA from *Z.mauritiana*, which contains high levels of alkaloids and lipids particularly in its leaves and roots. Thus, an efficient method for the isolation of high-quality total RNA from *Z.mauritiana* tissues was developed through the comparison and manipulation of CTAB based methods published and commercially available RNA extraction methods. Modifications in the extraction buffer by including PVPP, acid phenol and the addition of LiCl precipitation to the existing CTAB method produced the desired result. The modified CTAB-PVPP-LiCl method was then utilized for the isolation of high quality of RNA from various tissues of *Z.mauritiana* then used in downstream applications i.e RT-PCR, qRT-PCR analysis.

MATERIALS AND METHODS

Plant Material

Three months old *Z.mauritiana* plantlets were planted in an earthen pot (12 inch in diam) under greenhouse conditions under natural photoperiod and watered regularly. Thereafter heavy metal stress was imposed and continued for six months and control plants were grown in the pots containing normal soil. Six month old transplanted plants both control and metal stressed plants were uprooted carefully, throughly washed with sterile water and plant parts were separated (leaves, young shoot and roots) and were immediately immersed in liquid nitrogen and stored at -80°C until further processing. The entire process was done rapidly with atmost care to prevent RNA degradation.

RNA isolation protocol

All solutions except Tris buffer were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved. Glassware was baked for at least 4h at 180°C. Extraction buffer [2% cetyl trimethyl ammonium bromide (CTAB), 2% poly vinyl poly pyrrolidone (PVPP), 100 mM (hydroxymethyl) aminomethane (Tris–HCl) pH 8.0, 25 mM ethylene diamine tetra acetic acid (EDTA) and 2 M sodium chloride (Nacl) was autoclaved and, just before use of 2% β-mercapto ethanol (β-ME), 0.1% sodium dodecyl sulfate (SDS), 0.32M sodium acetate (NaOAc) were added to the extraction buffer and pre-heated to 65°C. Plant tissue (100mg) was ground to a very fine powder in liquid nitrogen using mortar and pestle. The powder was transferred to 2-ml micro- centrifuge tube containing 1 ml of pre-heated extraction buffer, vortexed and incubated at 65°C for 15 min with an intermittent shaking. 1 ml of phenol (saturated with Tris buffer with a pH of 6.7): chloroform mixture (1:1) v/v were added to the suspension and thoroughly shaken at 4^{o}C for 20 min. The suspension was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh tube (avoid contamination with interphase), to this equal volume of chloroform: isoamyl alcohol (49:1) was added, vortexed and centrifuged at 13,000 rpm for 15 min at 4^{o}C.The aqueous phase was transferred to another fresh tube and ice cold 8M LiCl was added to make final concentration of 3M. The sample was incubated at -80^{o}C for 30 min and the centrifuged at 13,000 rpm for 30 minutes at 4^{o}C.The supernatant was discarded and precipitate was washed with 100µl of 2M LiCl, 100µl of 75% ethanol was added to the precipitate and centrifuged at 13,000 rpm for 20 minutes at 4^{o}C. The pellet was air dried and dissolved in an appropriate volume of DEPC treated autoclaved water and store at -80°C.

RNA quantity and quality analysis

Purity and yield of total RNA isolated was assessed by determining the absorbance of the sample at 230 nm, 260 nm and 280 nm using a spectrophotometer (UV 1800 model Shimadzu Analytica Jena, AG, Germany). The A260/280 ratio was used to detect probable contamination with proteins and A260/230 ratio was used to check for carbohydrate contamination. The integrity of the RNA sample was analyzed on 1.5% denaturing formaldehyde agarose gel and stained with ethidium bromide [16]. The bands were visualized and documented the images using Gel documentation unit (G-BOX EF model, Syngene Ltd, UK).

First strand cDNA synthesis and semi quantitative RT-PCR

First strand cDNA was synthesized by reverse transcribing 2 µg of total RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific - India) in a 20 µl reaction using oligo(dT)$_{18}$ primers and RevertAid™ M-MuLV Reverse Transcriptase according to manufacturer's instructions. Reverse transcription was performed at 42°C for 1 hour followed by 70°C for 5 min to denature the RT-enzyme and cDNA was stored at −20°C until use. The synthesized cDNA was used in a reaction for PCR in order to estimate the expression level of the glutathione peroxidase using *GPX* (AB608053) specific primers FP (5'- ATGACTAGCCAGCCCAGATTC-3') and RP (5'- TCATGAGAGAGATTCCCAAG-3') to get an amplicon size of 510 bp. The following PCR program was used: 94°C for 3min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The program ended with a 8 min extension at 72°C. The amplified products were separated on a 1.2% agarose gel and visualized after staining with ethidium bromide and documented the images using Gel documentation unit (G-BOX EF model, Syngene Ltd, UK).

Quantitative RT-PCR analysis (qRT-PCR)

Quantitative Real Time PCR was performed for the stress specific gene glutathione peroxidase gene (AB608053) designing specific oligos for real time PCR FP (5'-GGATGCCAAGGGAGATGATA -3') RP (5-ACTGATTGCAAGGAAATGCC -3') and reference gene actin1 (EU251882) FP (5'-GTTACGCTCTACCTCACGCC-3') RP (5'-GAACAATTTCACGCTCAGCA-3') were selected and analyzed across different samples. qRT-PCR was performed on a Lightcycler 480 (Roche) with LightCycler®480 SYBR Green I Master (Roche). The reactions were performed according to the

manufacturer's instructions. The PCR program was initiated at 95°C for 5 min to activate *Taq* DNA polymerase, followed by 35 thermal cycles of 10 seconds at 95°C, 10 seconds at 55°C and 15 seconds at 72°C. Melting curve analysis was performed immediately after the real-time PCR. The temperature range used for the melting curve generation was from 55°C to 95°C. One template and non-template control and three biological replicates were utilized in the RT-PCR reactions. The determination of the crossing amplification point (Cp) as well as the relative quantification analysis (ΔΔCT-method) was performed using the Lightcycler 480 software v1.5. The amplification of non-template controls generated Cp values of above 45 and was not detectable. The non-normalized expression data were analyzed by geNorm v3.5 and Norm Finder v2.0 whereas the raw Cp values were imported into BestKeeper v1.0.

RESULTS AND DISCUSSION

The main objective of the present study was to develop an efficient and reproducible method for isolation of high quality RNA from an extremely recalcitrant xerophytic plant *Z.mauritiana* for subsequent functional genomics applications. The illustrated method in this manuscript is simple, reliable and has been reproduced successfully without appreciable difficulty. High quality RNA is important for functional genomics studies. Unfortunately, the task of extracting intact RNA is difficult due to the susceptibility of RNA molecules to the enzymatic degradation by RNase[3].Several existing methods use guanidinium thiocyanate for inhibiting RNase activity[3, 17] However, *Z.mauritiana* is attributed by a diverse group of secondary metabolites such as polyphenols, flavonoids, terpinoids, saponins and pectins[18] that co-precipitated with the GITC-phenol-chloroform buffers and failed to yield good quality of RNA[18]. Also commercial kits such as TRIzol reagent (Ambion, USA), NucleoSpin® RNA Plant (Macherey-Nagel, Germany), could not able to yield good quality of RNA when we tested. The methods described in the literature which were led to isolate high quality of RNA from recalcitrant tissue was only CTAB-based extraction buffer methods[5].Recently an improved method for isolation of high quality RNA from tea roots was developed by combining phenol-based protocols[19]. Since, soluble PVP is incompatible with phenol based buffers it was replaced with the insoluble PVPP for removal of polysaccharides and phenolic compounds and to prevent the oxidation of nucleic acids[19]. Application of the same method for isolation of RNA from the *Ziziphus* tissues yielded the RNA but with genomic DNA contamination and also with highly viscous polysaccharides which made RNA unsuitable for further functional genomics analysis. To overcome these problems the protocol developed here combines the use of an insoluble cross linked polymer polyvinyl-polypyrrolidone (PVPP), in the extraction buffer to exclusively precipitates polyphenol complexes by hydrogen bonding[20,21]. Addition of acidic phenol to the extraction buffer allowed efficient partitioning of RNA in the acidic aqueous phase leaving DNA in the basic phenolic phase, thereby preferentially separating RNA from the DNA and other impurities[8]. Besides, two precipitations with 8M LiCl which is a strong dehydrating agent and offers major advantages over other RNA precipitation methods in that it does not efficiently precipitate DNA, protein or carbohydrate[22].LiCl preferentially precipitates RNA from solution unless the DNA concentration during cold precipitation is high. Also it is the method of choice for removing inhibitors of translation or cDNA synthesis from RNA preparations[23].

The yield and purity of total RNA isolated from the leaves and roots by adopting present improved CTAB-PVPP-LiCl method produced highest RNA yield when compared with commercial kits, Tris-LiCl method and modified CTAB method (Table-1). The ($A_{260/230}$) and ($A_{260/280}$) ratios of the isolated RNA were 2.1±0.08 and 2.0±0.05, respectively, which indicated the RNA to be relatively free of proteins and contaminations (Table-1). The $A_{260/230}$ ratio was found higher than 2.0 indicating the lack of polyphenol and polysaccharide contaminations in the isolated RNA. The mean yield of RNA extracted by the method was 80µg/100 mg from leaf samples and 65µg/100 mg from the root tissue, with no

compromise on purity (Table.2). The ($A_{260/230}$) and ($A_{260/280}$) ratios of leaf RNA samples were 2.05±0.007 and root samples, 2.0±0.09 and 1.98±0.08 respectively, indicating presence of no protein, salt and solvents contaminants (Table-2). The quality of RNA was also checked by agarose gel electrophoresis using standard 1.5% of formaldehyde agarose gel (Fig 1). As evidenced from the picture, clear and distinct bands indicates that isolated RNA is highly integrated. Furthermore, the 28s rRNA band was equal to or more abundant than the 18s rRNA band indicating the isolated RNA was likely intact. In addition, there were no visible bands around the loading wells, indicating no gDNA contamination.

Table 1
Quality and yield of RNA extracted by different methods

Extraction method	RNA quality		RNA yield µg/100mg tissue
	$A_{260/230}$	$A_{260/280}$	
TRIzol Protocol	----------	----------	No yield
NucleoSpin® RNA Plant Kit	----------	----------	No yield
Tris-$LiCl_2$ method	1.68±0.03	1.98±0.05	20±1.54
Modified CTAB Protocol	2.07±0.04	1.92±0.02	45±4.5
Improved CTAB-PVPP-LiCl method	2.1±0.08	2.0±0.05	78±7.0

Table 2
Yield and quality of RNA from different tissues of Z.mauritiana using CTAB-PCPP-LiCl method

Tissues	RNA quality		RNA yield µg/100mg tissue
	$A_{260/230}$	$A_{260/280}$	
Leaf	2.05±0.07	2.0±0.08	80±4.04
Root	2.0±0.09	1.98±0.08	65±6.81

Agarose gel electrophoresis of RNA isolated from the leaves and roots of Z.mauritiana

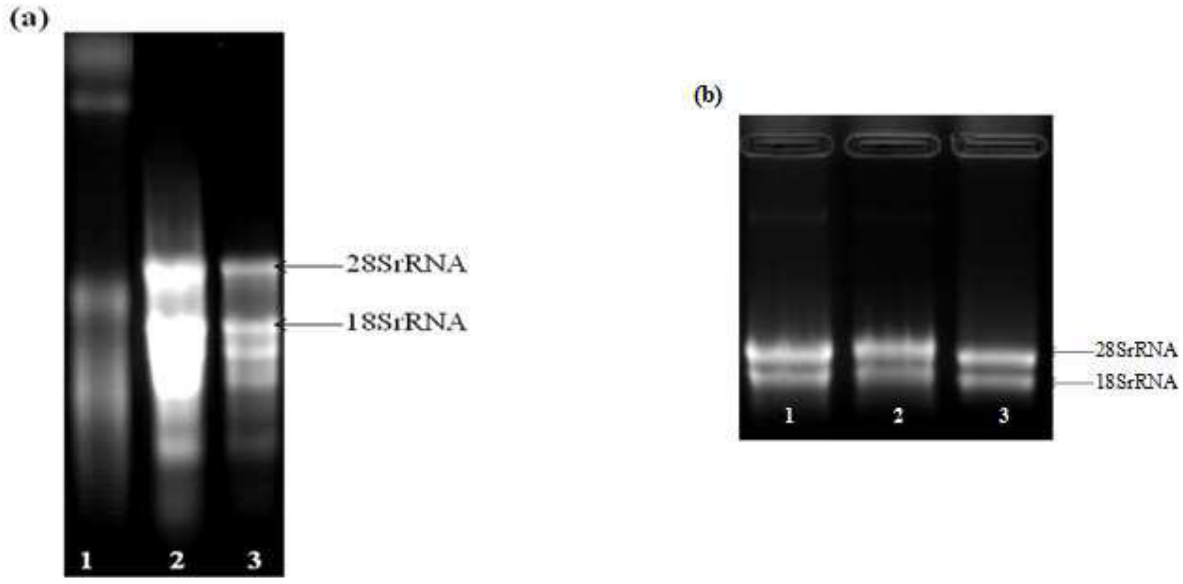

Figure1
Denaturing gel electrophoresis of RNA extracted from (a) leaves (b) roots using different methods: 1- Modified CTAB Protocol; 2- Tris-$LiCl_2$ method; 3-CTAB-PVPP-LiCl method

The suitability of isolated RNA for the functional genomics studies was demonstrated by semi-quantitative RT-PCR and qRT-PCR. Reverse transcription followed by standard PCR was used to test the RNA quality. After extraction of RNA from the roots and leaves of control and metal treated plants, cDNA was prepared by reverse transcription. Further, single standard cDNA was used as template in a standard PCR with gene specific primers to amplify an expected amplicon of 510 bp size of glutathione peroxidase (AB608053) (Fig 2). The total RNA isolated by this improved method was found reliable for RT-PCR as evidenced by the amplification of a *GPX* gene from the leaf and root tissue. Using total RNA isolated from leaves and roots of control and metal treated *Z.mauritiana* real time RT-PCR analysis was performed to monitor the glutathione peroxidase gene expression. As shown in Fig 3 the *GPX* gene expression was increased under metal treated conditions than the control leaf and root samples. These results indicated that total RNA samples isolated by this method were of high integrity, purity and suitable for downstream molecular analysis.

Semi quantitative RT-PCR analysis

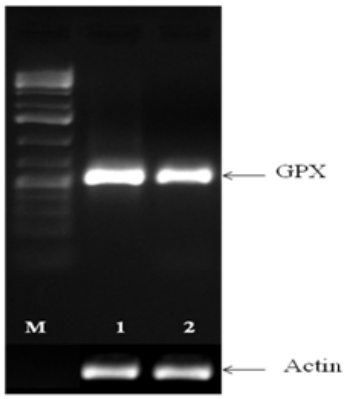

Figure 2
Semi quantitative RT-PCR analysis of GPX (glutathione peroxidase) gene (1) leaves (2) roots

GPX gene expression analysis

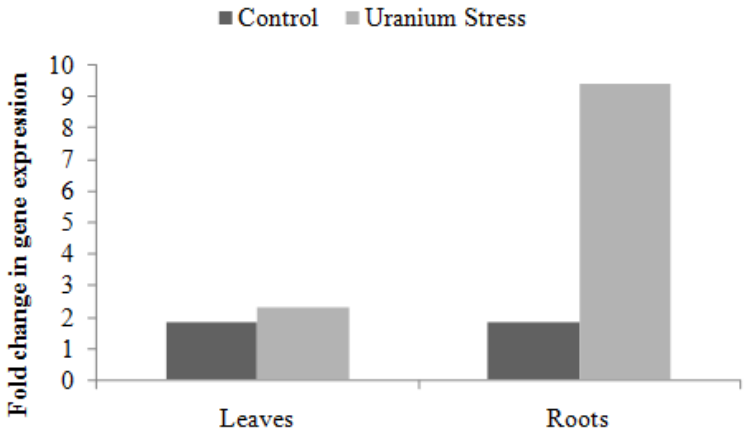

Figure 3
Quantitative RT-PCR analysis of GPX (glutathione peroxidase) gene

CONCLUSION

In conclusion this method is simple and effective in isolation of high quality of RNA from the leaves and root tissues of *Z.mauritiana,* from which otherwise was difficult using the conventional methods and commercial kits. The extracted RNA can be successfully used for downstream applications of functional genomics. This method is likely to be used in RNA extraction from roots and leaves of control and abiotic stressed woody plants where high polyphenol and other secondary metabolites content, hinder the process of RNA extraction.

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