

Original Research Article

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Isolation of RNA from Grains of Medicinal Rice “Njavara” using Improved TRIzol Method

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ABSTRACT

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Isolation of high-quality RNA from seeds is very critical for seed specific gene analysis. An investigation and comparison on the use of CTAB, SDS and TRIzol extraction procedures to extract high-quality RNA from grains of medical njavara rice were carried out in the present study. These protocols either failed to yield RNA or resulted in reduced yield with poor quality of RNA from Njavara rice grains. The starch and secondary metabolites present in njavara rice seeds hindered the isolation and the resuspension of precipitated RNA or contaminated the RNA pellets by co-precipitation. Hence, we modified the TRIzol RNA extraction protocol by addition of 0.5% N-lauryl sarcosine, 2% β -Mercaptoethanol and 1% PVP. Highly pure (A_{260}/A_{280} ratio ranged from 1.9 to 2.0) and intact RNA with a higher yield (up to 500 μ g/ml of RNA) could be obtained using modified TRIzol RNA extraction protocol. RNA obtained was testified and efficiently utilized for the cDNA preparation and amplification of *Ubiquitin* gene.

Introduction

Njavara rice, indigenous to Kerala, is a unique grain plant in the *Oryza* genus. It is used in the traditional medicine for the treatment of various diseases related to circulatory, respiratory and digestive ailments (Reshmi and Nandini, 2013). The nutritional and medicinal qualities of Njavara rice are mentioned in the ancient ayurvedic text “AshtangaHridaya”, circa 400-500 AD (Murthy, 2001). It is the only cultivar conventionally used in ayurvedic system of medicine in some specific treatments such as

Panchakarma in “Njavara Kizhi” and “Njavara Theppu”. Recently, it is reported that Njavara rice has also got anti-cancer properties and an anti-cancer gene associated with ‘Bowman-Brisk trypsin inhibitor protein’ has been identified (Shareesh, 2007; Shaliniet *al.*, 2012). The flavonoid compound present in njavara rice bran has also shown to have good antioxidant and anti-inflammatory activities (Bakiyalakshmi and Boominathan, 2014). Flavones, a chemotherapeutic agent belonging to a particular group of flavonoids, occur at higher concentration in Njavara rice and have also shown an anti-inflammatory

effect in carrageenan-induced rat paw edema (Mohanlal *et al.*, 2011).

Molecular and morphophysiological studies have shown that Njavara is a genetically distinct variety of rice with medicinal properties (Shareesh, 2007; Jose *et al.*, 2010; Kumar *et al.*, 2010). It is a short duration rice variety showing asynchronous flowering pattern with other commonly cultivated landraces that prevent cross-pollination and maintains its genetic purity (Sreejayan *et al.*, 2010). There are two types of Njavara rice based on the glume colour *viz.* black and yellow glumed. These two types are also described in ancient Ayurvedic text AshtangaHridaya (Sootrasthanam Chapter 6 sloka 7-10 Annaswaroopavigyani) (Joseph *et al.*, 2007). Black glumed njavara rice has red seeds with black-shaded grains. Yellow glumed njavara rice also has red colour seeds, but their grains are golden yellow.

The grains of black and yellow glumed njavara rice varieties are rich in flavonoids and other secondary metabolites. Extensive investigation is needed to understand the gene expression patterns and functions during grain filling to harvest stage in Njavara rice. Isolation of high-quality RNA lacking polysaccharides, proteins, phenolic compounds, genomic DNA or secondary metabolites is crucial to undertake the analytical techniques like reverse transcription-polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR) and complementary DNA (cDNA) library construction etc. Hence, in the present study, an attempt was made for developing a protocol for the extraction of good quality RNA from the grains of black and yellow glumed njavara rice varieties by comparing and modifying the different reported protocols. Njavara rice seeds are rich in high levels of polysaccharides, lipids, proteins and phenolic compounds. Phenols are reported to

reduce the yield and quality of RNA (Tai *et al.*, 2004; Groppe and Morse, 1993; Vergara and Ismail, 2007). So far there are no protocols reported for isolating RNA from rice seeds of black and yellow glumed njavara. Plant species, organs and tissues of plants respond differently to the RNA extraction protocols due to the differences in their metabolic activities (Jhala *et al.*, 2015). Hence, in the present study, we compared three protocols based on CTAB, SDS and TRIzol, along with a modified TRIzol method to establish an effective protocol to obtain high quality of RNA with high yield from njavara seeds.

Materials and Methods

Two types of Njavara medicinal rice namely, black glumed njavara and yellow glumed njavara were grown in pots at the Department of Plant Physiology, College of Agriculture, Vellayani (8°5'N and 76°16' E) and the grains were collected at grain filling stage of the crop.

Solutions and reagents

All the reagents used in the study were of molecular biology grade. Gloves were used at all times during the preparation and changed frequently. Diethyl pyrocarbonate (DEPC) treated water (DTW) (0.1%) was used for preparing the solutions. Disposable pipette tips and centrifuge tubes were autoclaved at 121°C for 40 min without diethylpyrocarbonate (DEPC) treatment. Porcelain mortars, pestles and metal objects were wrapped in aluminium foil and baked at 180°C for more than 5 h. Plastic and glassware were immersed in 0.1% (v/v) DTW at 37°C overnight and then autoclaved at 121°C to inactivate RNases. Mortar and pestle were chilled and wiped with RNAase zap to remove any traces of RNAase before using them to grind the grain samples.

CTAB RNA extraction protocol (Gambino *et al.*, 2008)

Grains (100 mg) were ground to a fine powder and collected in RNase-free tubes. The pre-warmed CTAB extraction buffer (1 ml) and 20 μ l β -Mercaptoethanol (β -ME) was added and mixed by vortexing. CTAB extraction buffer included 2% CTAB (W/V), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 2% PVP. The samples were incubated at 65°C for 20 minutes and vortexed every 5 minutes. An equal volume of chloroform-isoamylalcohol (24:1) was added after incubation. It was mixed and centrifuged for 10 min at 15,000 g at 4°C. The aqueous phase was transferred to a fresh tube and extracted again with an equal volume of chloroform-isoamyl alcohol as before. One-third volume of 8 M lithium chloride (LiCl) was added to the supernatant and kept overnight at -20°C. RNA was pelleted by centrifugation for 10 min. at 15,000 g at 4°C. The RNA pellet was washed with 75% ethanol and dried at room temperature and subsequently resuspended in 50 μ l ddH₂O.

SDS RNA extraction protocol (Ekatpureet *al.*, 2019)

Grains (100 mg) were taken in chilled mortar and ground to a fine powder using liquid nitrogen. Extraction buffer (3 mL) was added to homogenate and incubated in a hot water bath for 30 minutes at 65°C. Extraction buffer contained 150 mM Tris base (hydroxymethyl - hydrochloride), 4% (w/v) SDS, 100 mM EDTA (pH 7.5, adjusted with saturated boric acid), 60 μ l β -ME (2%, v/v) and 3% (w/v) PVP-40. The homogenate (approx. 750 μ l) was transferred to RNase free 2 ml tube and precipitated using 5 mM potassium acetate (66 μ l) and absolute ethanol (150 μ l). Then an equal volume of chloroform-isoamyl alcohol (49:1, v/v) was added and vortexed briefly and centrifuged at 16,000 g for 20 minutes at room temperature. The supernatant

was carefully transferred to a fresh tube and this step was repeated again to remove any suspended cell debris. The supernatant was taken in a fresh tube, added equal volume (850 μ L) of phenol:chloroform: isoamylalcohol (25:24:1) and vortexed for 10 seconds and then centrifuged at 16,000 g for 15 minutes at room temperature. The supernatant was collected in a fresh tube and 850 μ L of chloroform-isoamyl alcohol (49:1, v/v) was added. The tube was briefly vortexed for 10 seconds and centrifuged at 16000 g for 15 minutes at 4°C. The supernatant was once again collected and 3 M LiCl was added to it and then mixed by inversion and kept overnight at 4°C. The content was then centrifuged at 16,000 g for 20 minutes at 4°C. Pellet recovered at this stage was washed twice with 70 percent ethanol. Then the pellet obtained was air-dried at room temperature under sterile laminar airflow and resuspended in 30 μ L of DEPC-treated sterile distilled water. RNA was quantified in a UV-light spectrophotometer and stored at -80°C until use.

TRIzol method (Chomczynski and Sacchi, 2006)

Grain samples (100 mg) were ground into a fine powder using liquid nitrogen. 1 ml of TRIzol reagent was added to the powdered samples and mixed gently to homogenize the mixture and incubated at ambient temperature for 5 minutes. Then, the mixture was transferred to a pre-chilled microfuge tube and 0.2 ml chloroform was added. After vigorous shaking, for about 15 seconds it was incubated for 5 minutes at room temperature. The microfuge tubes were kept in ice for 10 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase from the tubes was transferred to fresh microfuge tubes. Hundred percent ice-cold isopropanol of 0.5 ml was added to each tube and kept for incubation at room temperature for 10 minutes. Then the contents in the tubes were

mixed by inverting the tube slowly and then again centrifuged at 12,000 g for 10 minutes at 4°C. After that, the supernatant was discarded carefully and the pellet was washed with 1 ml of 75% ethanol prepared in DEPC treated water and spun at 7,500 g for 5 minutes at 4°C. Then the supernatant was removed and the pellet was dried in the laminar airflow chamber. The pellet was then dissolved in 30 µl of RNAase free water and incubated for 10 minutes at 55-60°C. The isolated RNA was stored at -80°C for further use.

Modified TRIzol method

The ground powder of grains (100 mg) powder was transferred quickly to a 1.5 ml tube with 1 ml TRIzol reagent, 0.5% N-lauryl sarcosine, 2% β-ME and 1% PVP, mixed completely by vortexing the tube and incubated for 10min at room temperature. Then, 0.2 ml chloroform was added and vortexed for about 15 seconds. It was incubated for 5 minutes at room temperature and then centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase from the tubes was transferred to fresh microfuge tubes. RNA was precipitated with 0.5 ml ice-cold isopropanol for 10 minutes at room temperature and centrifuged at 12,000 g for 10 minutes at 4°C. The pellet obtained was washed with 1 ml of 70% ethanol prepared in DEPC treated water and sedimented at 7,500 g for 5 minutes at 4°C. It was then dried in a laminar airflow chamber and dissolved in 30 µl of RNAase free water and incubated at 55-60°C for 10 minutes. The isolated RNA was stored at -80°C for further use.

Qualitative and quantitative analysis of RNA

The quality of RNA was determined by using agarose gel electrophoresis. The gel was prepared using 1.5 g of agarose powder dissolved in 100 ml of 1 X TBE. It was

cooled down to about 60-65°C and ethidium bromide (EtBr) was added to it. RNA samples (5 µl) were loaded in the wells carefully. The voltage was maintained at 5 V/cm and the gel was allowed to run till the RNA reached three fourth of gel. The bands were visualized and documented in Gel Doc Unit (Bio-Rad) using Quantity One software.

RNA quantification was done by taking absorbance in UV-visible spectrophotometer (Model-ELICO SL 218, Double Beam, UV-VIS, Spectrophotometer, India) at the wavelength of 260 and 280 nm. The absorbance value of 1.0 at 260 nm indicated that 40 ng µl⁻¹ of RNA was present in the samples. The concentration of RNA in the sample was determined by the formula:

$$\text{Concentration of RNA (ng } \mu\text{l}^{-1}) = A_{260} \times 40 \times \text{Dilution factor}$$

Where, A_{260} = Absorbance at 260 nm

The quality of the RNA samples was known from the ratio of the OD values recorded at 260 and 280 nm. The best quality of RNA was referred to by A_{260}/A_{280} value between 1.8 and 2.

Preparation of cDNA

RNA isolated from improved TRIzol method was used for the synthesis of cDNA. The cDNA was synthesized using “Thermo Scientific Verso cDNA Synthesis Kit” according to the protocol provided by manufacturers. The kit contained verso reverse transcriptase which could generate long cDNA strands, RNAase inhibitor to protect RNA templates from degradation and RT enhancer which help to remove DNA contamination. The kit also contained oligo dT primer and Random hexamer. The contents were mixed gently and incubated at 42°C for 30 minutes followed by another incubation at 92°C for 2 minutes and then the

cDNA samples were stored at -80°C for further analysis.

Qualitative analysis of cDNA

The cDNA synthesis was confirmed by standard PCR technique using housekeeping

gene *Ubiquitin (UBQ5)* with gene-specific primers. The final amplified PCR product was separated on agarose gel (1.2%) and documented using Gel Doc Unit (Bio-Rad) using Quantity One software. The details of primer used (Park *et al.*, 2016) was as follows

Genes	Locus ID	Forward (5' to 3')	Reverse (5' to 3')
<i>UBQ5</i>	Os01g0328400	GAAGTAAGGAAGGAGG AGGA	AAGGTGTTTCAGTTCC AAGG

Results and Discussion

Our initial attempt using the three existing methods (CTAB, SDS and TRIzol) to extract total RNA from the plant tissues rich in polysaccharides, polyphenols and protein yielded variable and unsatisfactory results (Table 1 and Figure 1). The yield and quality of total RNA obtained were unexpectedly lower (Table 1). RNA obtained from the CTAB- based method (Gambino *et al.*, 2008) and SDS method (Ekatpure *et al.*, 2019) was found highly degraded and impure. The pellet formed was not completely soluble and RNA recovery was very poor. In addition, there was serious genomic DNA contamination to the isolated RNA. Moreover, the entire procedure was time consuming and labour intensive. With TRIzol method, intact RNA could be obtained, but the quantity obtained was not sufficient and the quality did not qualify for its use in transcriptional level studies. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of RNA (Table 1). Clearly, the efforts with these existing methods were not successful. But among the three methods, TRIzol method was found better as it gave comparatively good recovery. Considering the purity ratio (A_{260}/A_{280} : 1.8 to 2) and the quantity of RNA, further modifications were made in TRIzol method to get better results.

TRIzol (Chomczynski and Sacchi, 2006) is a

favourite reagent to extract RNA from different plant materials for the advantage of being simple, relatively short and easy to operate. Total RNA extracted from Njavara rice seeds using TRIzol method gave a low quantity of RNA with a lot of impurities and no DNA contamination. The impurities might be related to the high quantity of carbohydrates and secondary metabolites present in the grains. The compounds like carbohydrates, polyphenols and protein bind or co-precipitate with RNA which degrade the quality and quantity of extracted RNA (Helen *et al.*, 2004; Asif *et al.*, 2006; Wang *et al.*, 2008; Shu *et al.*, 2014). Another problem is the degradation of RNA by ribonuclease (RNase) enzyme. RNase is very stable and difficult to inactivate. Heat can denature ribonuclease but when the temperature is lowered, the enzyme spontaneously refolds into its native active conformation. TRIzol is constituted of guanidine thiocyanate, a strong chaotropic denaturant which completely inactivates the ribonuclease present in the material to be extracted. To strengthen TRIzol-lauryl sarcosine (0.5%). β -Mercaptoethanol (2%) and PVP (1%) were added. PVP aids in the removal of polyphenols (Salzman *et al.*, 1999). It unfolds the RNase at an exceedingly higher rate than the rate of RNA hydrolysis. β -mercaptoethanol is a reductant which breaks disulfide bonds, thereby facilitating irreversible denaturation of the enzyme (Rashid *et al.*, 2016). N-

lauroylsarcosine is an anionic surfactant and does not produce excess foaming. It denatures the ribonuclease, inhibit its active site, ensures maximum solubility of nucleic acids and removal of interfering contaminants (e.g. polysaccharides, polyphenols). The chemical agent, diethylpyrocarbonate (DEPC) used to treat plastic wares and water destroys the ribonuclease and translational activity.

Improved TRIzol method developed was

found to be rapid and effective in isolating high-quality RNA from Njavara grain samples of 100 mg. The quantity obtained was almost double with high purity (Table 1). The quality of the RNA extracted by this method was demonstrated by intact sharp 28S and 18S rRNA bands and the lack of RNA degradation on agarose gels (Figure 1). The A_{260}/A_{280} ratio ranged from 1.9 to 2.0, indicating a lack of protein, polysaccharides or polyphenols contamination (Table 1).

Table.1 Quality and quantity of total RNA extracted by four methods from two Njavara medicinal rice grains

	Methods used	A_{260}	A_{280}	A_{260}/A_{280} Ratio	Quantity ($\mu\text{g/ml}$)
Black Njavara	CTAB	0.011	0.007	1.57	132
	SDS	0.012	0.007	1.7	144
	TRIzol	0.017	0.01	1.7	204
	Modified TRIzol	0.041	0.021	2.0	492
Yellow Njavara	CTAB	0.009	0.006	1.5	108
	SDS	0.011	0.007	1.57	132
	TRIzol	0.019	0.011	1.7	228
	Modified TRIzol	0.034	0.018	1.9	408

Fig.1 Electrophoresis of total RNA isolated from grains of medicinal rice. A: CTAB Method; B: SDS method; C: TRIzol Method; D: Modified TRIzol method; Lane 1: Black glumed njavara rice; Lane 2: Yellow glumed njavara

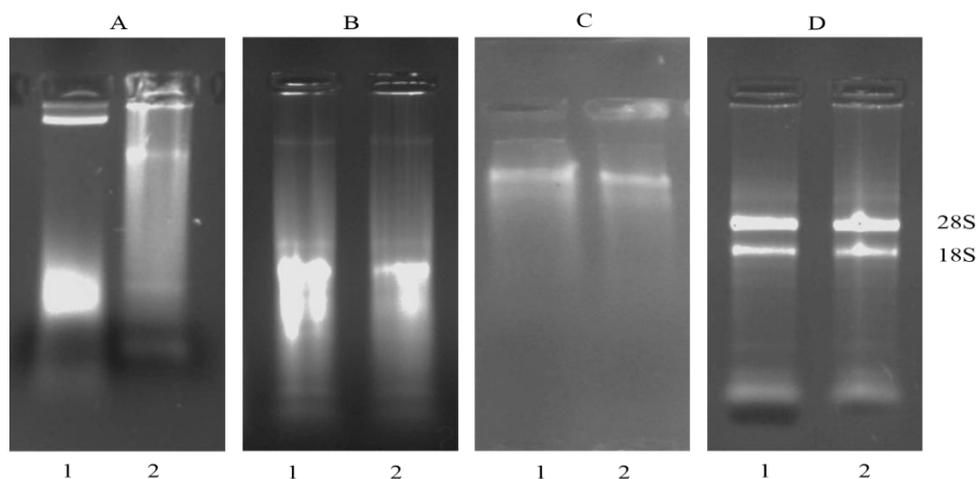
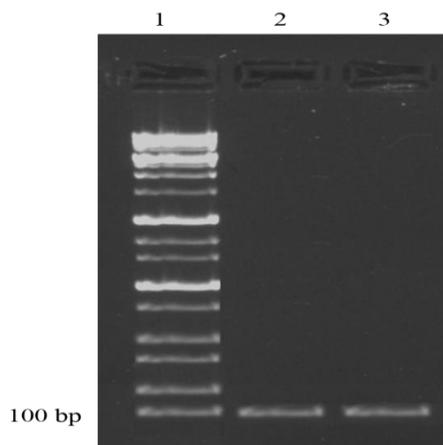


Fig.2 Electrophoresis of PCR products with *Ubiquitin (UBQ5)* specific primers using cDNA generated from total RNA isolated using modified TRIzol method. (Lane 1: Ladder (100 bp); Lane 2: Black glumed Njavara; 3: Yellow glumed njavara)



To evaluate the integrity of the extracted RNA, cDNA was prepared and used to amplify a housekeeping gene, *Ubiquitin*. The synthesis of cDNAs using the extracted RNA and reverse transcriptase enzyme was successful. The target band with expected size of 100bp was obtained (Figure 2). These results indicate that the improved TRIzol method standardized in this study is effective in the extraction of total RNA for the transcript analysis of gene expression in the grains of njavara rice.

In conclusion these report an improved TRIzol method for high-quality and quantity RNA extraction from black and yellow glumednjavara grains which are rich in starch and secondary metabolites. This method can also be applied to extract RNA from grain/seeds rich in polyphenols and polysaccharides. The effectiveness of this improved method to extract total RNA from grains of njavara rice was confirmed with RT-PCR of *Ubiquitingene*.

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Author's Contributions

This work was carried out in collaboration with all authors. Author Wagh conducted the research work, interpreted the data and prepared the manuscript. Author Viji helped to design the experiment corrected manuscript thoroughly. Authors Dr. Soni, Dr. Jadhav, Mr. Ekatpure, Dr. Roy, Dr. Manju and Dr. Beena helped author Wagh Y.S during the experiment and contributed in the manuscript. All authors read and approved the final manuscript.

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