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Improved Protocol of RNA Isolation for Transcriptome Analysis of Poaceae Plants

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ABSTRACT

The existence of metabolites that interfere with isolation procedures and downstream applications makes plant RNA extraction difficult. The current research used a standardized RNA extraction protocol from maize (*Zea mays* L.). We developed a protocol for extracting pure RNA from plant tissues using both extraction buffer and the TRIzol reagent, and show that this RNA extraction method works not only at Low temperatures but also at room temperatures, making it the easiest and most efficient method for extracting pure and undegraded RNA directly from tropical plants in the field. RNA isolation methods based on our modified protocol yielded good results in maize leaf, seed, flowers and other grass species. The isolated RNA was found to be suitable for both PCR and RT-PCR amplification and transcriptome analysis. The method is repeatable and can be used to isolate high-quality RNA and conduct gene expression studies. RNA extraction paves the way for deciphering the complex regulatory network involved in multiple stress responses by studying gene-environment interactions at the transcriptome level.

HIGHLIGHTS

- This study provides simple and rapid protocol for RNA extraction from maize and Poaceae grasses.
- The isolated RNA shows good quality and concentration.
- BioAnalyzer quantification provided good RIN value, which shows utility for various downstream applications like transcriptome analysis.

Keywords: RNA extraction, Poaceae plants, TRIzol reagent, downstream applications

Plant molecular biology has exploded in popularity in recent years. In many crop-breeding programs, molecular markers are now commonly used to map loci and genome regions (Varshney *et al.* 2006). The number of PCR-based markers and gene expression studies is growing at an exponential rate. In these studies, high-quality, intact RNA is essential for conducting PCR and RT-PCR experiments (Shu *et al.* 2014). However, due to differences in metabolic activities, individual organisms, organs, and/or tissues of plants can behave differently during RNA extraction.

Polysaccharides are abundant in maize (*Zea mays* L.). Which are known to inhibit polymerase activity and co-precipitate during RNA extraction (Jhala *et al.* 2015; Wang *et al.* 2012; Xu *et al.* 2010; Gambino *et al.* 2008; Wan and Wilkins 1994; Fang *et al.* 1992). A successful extraction protocol should be easy to follow and produce good results. The RNAzol RT reagent process, CTAB-based method, Direct-zol™ RNA miniPrep kits-based protocols, modified CHAN method, SDS (Sodium dodecyl sulphate) and GT (Guanadine thiocyanate), extraction buffer with TRIzol reagent methods were used by the researchers to isolate complete RNA free from

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polysaccharide and polyphenolics rich plants such as rice, mango, banana, maize, pecan, pear leaves, grapes and potato tubers (Liu *et al.* 2018, Breitler *et al.* 2016; Jhala *et al.* 2015; Wang *et al.* 2012; Xu *et al.* 2010; Gambino *et al.* 2008; Rodrigues *et al.* 2007).

The quality of the RNA molecule must be consistent to conduct subsequent gene expression experiments. The TRIzol reagent (TRIzol T-924) is a ready-to-use reagent for extracting complete RNA from plant cells and tissues. TRIzol, a reagent made consisting of a mono-phasic solution of phenol and guanidine isothiocyanate, was invented by Chomczynski and Sacchi in (1987). The effectiveness of the procedures is determined by the chemical properties of the substance being used. For an effective extraction technique to isolate RNA, Jhala *et al.* (2015) RNA extraction protocol with some improvements is used.

For the RNA isolation, we have performed different available protocols from the literature but couldn't get desirable results. Therefore in this study, we have standardized a high-quality RNA isolation protocol from maize and grasses of the family Poaceae.

MATERIALS AND METHODS

Plant material

The maize was collected from the village of Munjka, Rajkot (22.2931° N, 70.7436° E) and other grasses were collected from the Kutch desert (23.7337° N, 69.8597° E) Gujarat, India. Then it was deposited in PPMB lab, Department of Biosciences, Saurashtra University Rajkot. To obtain fine powder for RNA extraction, plant organs (leaf, seed and flower) were quickly ground in liquid nitrogen.

Modified RNA isolation protocol

The sample and lab ware were washed with 0.1% DEPC water. Approximately, 100mg plant material crushed in liquid nitrogen to fine powder and extraction buffer contained 150 mM Tris base, 1% (w/v) SDS, 100 mM EDTA (pH 7.5), 1% β-Mercaptoethanol and 1% (w/v) PVP-40. This mixture was centrifuged at 13000g for 8 min. The supernatant was transferred and 1 ml of Trizol (Sigma) was added in a 2ml Eppendorf tube. It was vortexed at room temperature (R.T.) for 5 min. The mixture was centrifuged at 13000 g for 10 min at 4°C. The supernatant was transferred in 2ml Eppendorf (treated in 0.1% DEPC) tube. 200 µl chloroform was added and tubes were shaken vigorously by hand for 15 sec and incubated for 5 min at R.T. The sample was again centrifuged at 13000g for 15 min at 4°C. The aqueous phase was separated and an equal volume of Phenol: Chloroform (P:C) was added and centrifuged at 10000g for 12 min at 4°C. The aqueous phase was transferred to a fresh tube and 1/10 volume of 3 M sodium acetate (pH 4.8, 40 µL) and two volumes of ice-cooled ethanol and incubates at R.T. for 10 min for the precipitation of RNA. This was followed by centrifugation at 13000g for 10 min at 4°C. The RNA forms a pellet on the side and bottom of the tube, white to brown in colour. The supernatant was removed and the RNA pellet was washed with 75% ethanol and mixed by vortexing, again centrifuged at 7,400 g for 5 min at 4°C. All traces of ethanol were removed and leave tubes open in a laminar airflow chamber; the pellet changed from white to clear. The pellets were dissolving in 100 µl RNase-free MQ (DEPC Treated water). Obtained RNA Samples were stored at -20°C.

The quality of total RNA and cDNA synthesis

The quality of total RNA was assessed using the RNA6000 Nano Chip in an Agilent 2100 BioAnalyzer (Agilent Technologies, CA, USA). To calculate an RNA integrity number (RIN) value, utilize the Agilent 2100 Bioanalyzer Expert Software™. The Applied Bio-systems® High capacity cDNA transcription kit was used to make the cDNA, which was then processed according to the manufacturer's instructions.

cDNA quantification

The µQuant microplate reader, Bio-Tek instruments integration, USA, was used to determine the consistency and quantity of the RNA extracts. At A260 nm, the concentration was calculated. The ratios A260/A230 and A260/A280 were used to assess contamination due to phenol/carbohydrate and proteins, respectively (Sambrook and Russell 2001).

Downstream application

Two methods were used to assess the consistency of the extracted RNA: gel electrophoresis and

PCR amplification. The RNA samples were first denatured with a 1% urea treatment before being combined with gel loading dye at room temperature for 10 minutes. The samples were loaded onto a 1% agarose gel and visualized with UV light after being stained with ethidium bromide. Extracted RNA was converted to cDNA and amplified with ADP-*glucose pyrophosphorylase small subunit (OsAGPS1)* F: GTGCCACTTAAAGGCACCATT R: CCCACATTTCAGACACGGTTT, 52.9 Ta (°C) to verify the method's efficiency and reliability. The total volume of the PCR reaction mixture was 20 µl, and it is carried out in three steps, with primer annealing at Ta.

RESULTS AND DISCUSSION

However, isolating RNA from plant tissues can be difficult mainly because (Asif *et al.* 2006), the interphearance of secondary metabolites during extraction. Some plant tissues, such as fruits, storage tubers, flowers, and seeds, contain a high concentration of storage proteins, polysaccharides, polyphenolics, and other secondary metabolites that would co-precipitate with the RNA and pose a significant barrier to RNA isolation (Liu *et al.* 2018; Sangha *et al.* 2010; Isaacson *et al.* 2006). For RNA extraction, methods based on TRIzol and/or isothiocyanate have been commonly used. These techniques are commonly used in a variety of systems, including plants, animals, bacteria, and viruses. Cereal crop endosperm, on the other hand, contains a lot of starch, which allows samples to solidify in RNA extraction buffers. Some plant tissues with a high level of starch may prevent precipitated RNA from being resuspended and contaminate it due to co-precipitation, affecting its storage, quality and quantity of isolates (Wagh *et al.* 2020).

Several methods for RNA isolation were tested in this study (Liu *et al.* 2018; Breitler *et al.* 2016; Jhala *et al.* 2015; Wang *et al.* 2012) and Direct-zol™ RNA miniPrep kit; the one that was most commonly used in laboratories was the TRIzol reagent-based methods, while the other was extraction buffer combined with the TRIzol reagent-based protocol. In many plant systems, extraction buffers made with detergents like SDS have shown to be more effective (Mandaliya *et al.* 2010). EDTA has an affinity for divalent ions like Ca^{+2}, Mn^{2+}, and Mg^{2+}, it was used to cure the damaged cell. EDTA is primarily utilized in extraction buffer to chelate magnesium ions, which is a required co-factor for nucleases (AbouHaidar and Ivanov 1999). When extracting RNA from plant crude extracts, DNA, protein, pigments, and polysaccharides are all present in considerable amounts (Jhala *et al.* 2015). To eliminate significant protein phenolic and cell debris impurities, phenol/chloroform extraction was required (Rubio-Piña and Zapata-Pérez 2011). Using phenol/chloroform, remaining proteins and other cellular impurities were removed or isolated from the RNA. On a 1% gel electrophoresis of selected samples, our method developed distinct bands.

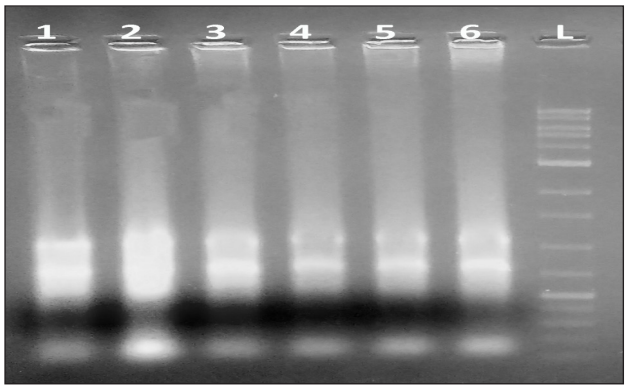

Fig. 1: RNA extraction in a laboratory with recommended cold conditions to isolate total RNA using Direct-zol™ RNA miniPrep kits-based protocols, electrophoresis separation using 1% agarose gel of RNA extracted from the three *Zea mays* samples (Lane: 1&2 is a mature leaf, Lane: 3&4 is a flower, Lane: 5&6 at seeds, Lane: L is ProxiO 1 kb leader)

Evaluation of the quality of RNA extraction in maize plant

We compared RNA extracted from the same samples in the lab using TRIzol reagent or the commercial Direct-zol™ RNA miniPrep kit with RNA extracted from the same samples in the lab using TRIzol reagent or the commercial Direct-zol™ RNA miniPrep kit to test the efficacy of TRIzol reagent isolate RNA samples in maize plant tissues. When complete RNA was isolated from maize plant tissues in the lab using the TRIzol reagent, it is not well suited to extracting RNA (from maize plant materials), produced the poorest results. The majority of small RNAs are lost due to contamination with genomic DNA (figure 1-3). In another method of RNAzol RT reagent method (Breitler *et al.* 2016) four samples of maize, one sample of grass (*Cymbopogon citratus*) and crop

plant wheat are considered. In this, all samples provided smeared band with little genomic DNA contamination (Fig. 2).

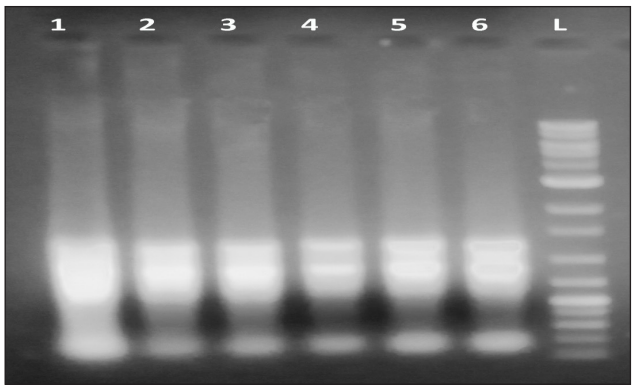

Fig. 2: Total RNA from maize leaf using RNAzol RT reagent method (Breitle *et al.* 2016 protocol). *Zea mays* (Lane: 1 is mature flag leaf, Lane: 2 is a flower, Lane: 3&4 are seeds), Grasses (Lane: 5 is *Cymbopogon citratus* leaf), *Triticum aestivum* (Lane: 6 is a leaf), Lane: L is ProxiO 1 kb leader. The isolated RNA was analyzed by electrophoretic separation using 1% agarose gel

In RNA extraction buffer ((100mM Tris-HCl (pH 9.0), mixed with 1% β-mercaptoethanol (v/v)) with TRIzol reagent (Jhala *et al.* 2015) four samples of *Zea mays*, one sample of grass (*Cymbopogon citratus*) and *Triticum aestivum* are considered. Here in all samples, moderate bands were observed but in this genomic DNA contamination was very high (Fig. 3).

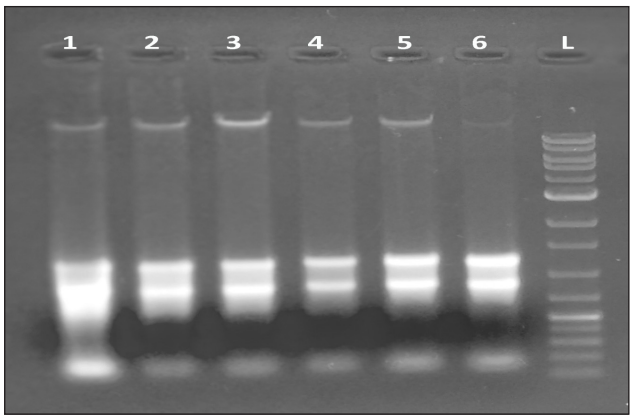

Fig. 3: Total RNA from using extraction buffer with TRIzol reagent (Jhala *et al.* 2015 protocol): *Zea mays* (Lane: 1 is mature flag leaf, Lane: 2 is a flower, Lane: 3&4 are seeds), Grasses (Lane: 5 is *Cymbopogon citratus* leaf), *Triticum aestivum* (Lane: 6 is a leaf), Lane: L is ProxiO 1 kb leader, on 1% agarose gel electrophoresis

A reliable method applicable to several organs

To extract pure and underestimated RNA, the simplified TRIzol reagent RNA isolation protocol was first adapted to the maize plant using mature Flower, leaf, and seed samples (Fig. 4).

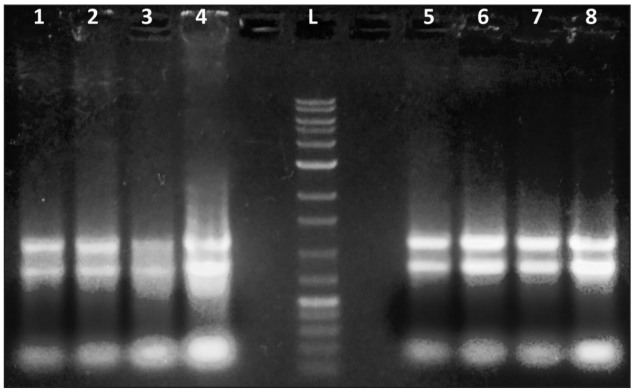

Fig. 4: Total RNA extracted with our modified protocol from maize, wheat and grasses: grasses (Lane: 1 is *Cynodon dactylon*, 2 is *Cymbopogon citratus* and 3 is *Panicum maximum*), *Triticum aestivum* (Lane: 4 is a leaf), Zea mays (Lane: 5 and 6 are seeds 1&2, Lane: 7 is a flower, Lane: 8 at leaf), Lane: L is ProxiO 1 kb leader, on 1% agarose gel electrophoresis

RNA samples with an RNA integrity number >10, as assigned by the BioAnalyzer which ranges from 5.80 to 8.50 (Table 1).

Table 1: The quality of total RNA by RIN value (RNA integrity number) and RNA concentration from Bioanalyser 2100

Sample	RIN value	Concentration RNA ng/µl
Zea mays L. seed 1	8.50	1,974
Zea mays L. seed 2	8.20	1,922
Zea mays L. leaf	5.80	1,492
Zea mays L. flower	6.80	768

In electrophoresis using, BioAnalyzer showed three distinct RNA bands (Fig. 5). It was noted in other's reports that the RIN value >5 is considered good for downstream applications (Fleige and Pfaffl 2006). With an A260/A280 ratio of 1.79 to 1.92, an electrophoretic separation agarose gel electrophoresis using eight samples reported good RNA quality. The absence of genomic DNA in the RNA samples was verified by agarose gel electrophoresis. The cumulative RNA derived from 100 mg of fresh maize leaf tissues amounted to 593.08 ng/µl on average. The A260/A280 ratio of RNA extracted from more difficult plant-related samples, such as seeds and flowers, was 1.80 and 1.85 (Table 2).

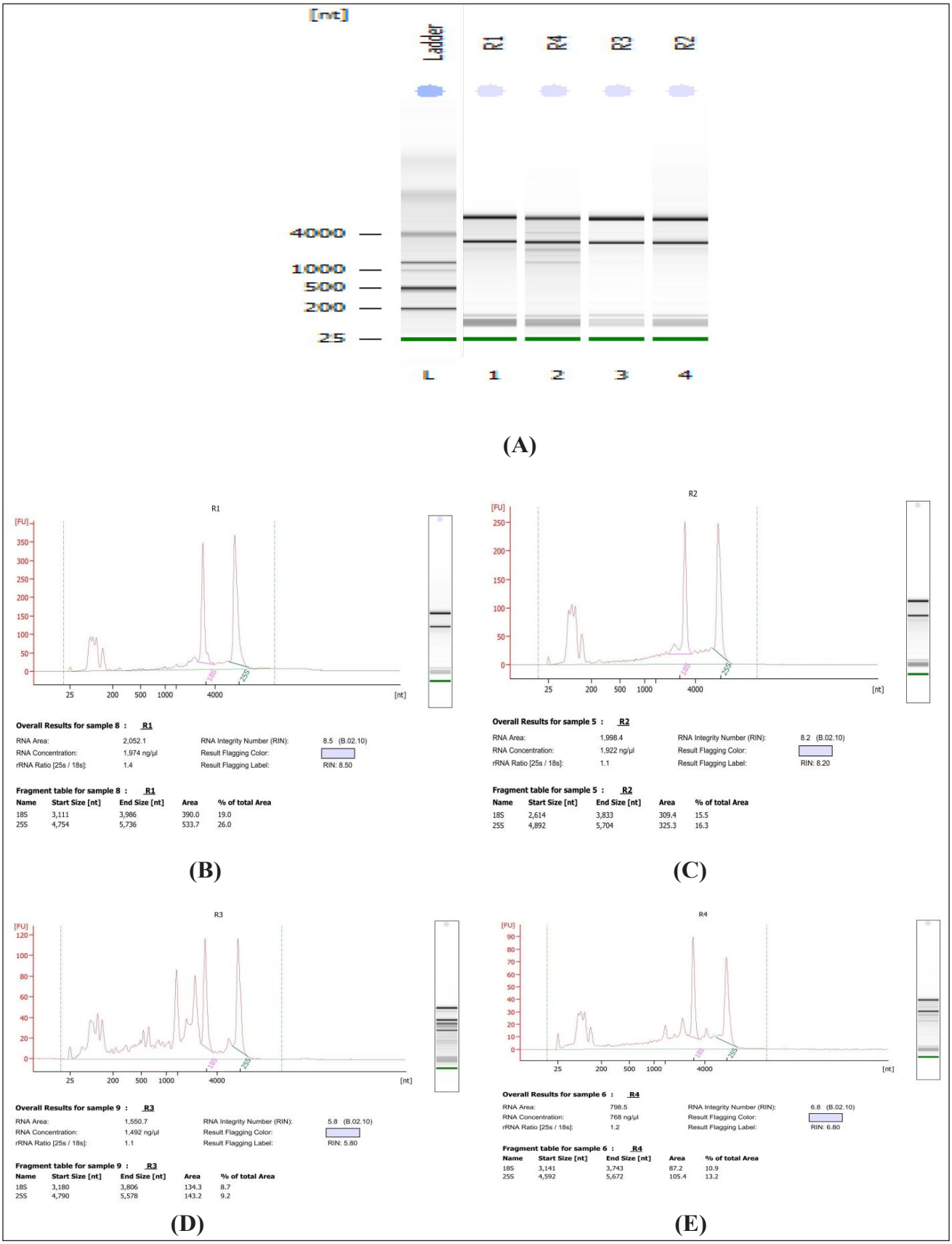

Fig. 5: (A) In electrophoresis using BioAnalyzer showed three distinct RNA bands from *Zea mays* L. plant organ (Lane: L is Leader; Lane: 1 is Flower; Lane: 2 is Leaf; Lane: 3 is Seed1 and Lane: 4 is Seed 2). **(B, C, D, E)** An electropherogram of various maize plant organs

Table 2: Eight samples for cDNA quantification, their purity and concentration

Sample	Purity 260/280 ratio	Concentration cDNA ng/µl
Zea mays L. seed 1	1.80	555.50
Zea mays L. seed 2	1.80	493.16
Zea mays L. leaf	1.82	593.08
Zea mays L. flower	1.85	564.28
Triticum astivum	1.83	1031.19
Cynodon dactylon	1.79	292.33
Cymbopogon citratus	1.82	298.09
Panicum maximum	1.92	450.63

A useful method for tropical crops (wheat and other grasses)

Other agronomically important crops were considered in this study to confirm the importance of this RNA extraction method for tropical plants. Complete RNA was extracted from mature leaf samples collected in the field from, wheat and other grasses using the protocol developed for maize tissue. Agarose gel electrophoresis was used to examine the extracted RNAs (Fig. 4). Both the quality and quantity of extracted RNAs were high, similar to maize, demonstrating the method's versatility for tropical dicots and monocots.

For maize flowers, seeds, and leaves with high levels of starch, a modified TRIzol reagent RNA extraction protocol was found to be efficient and accurate. The method used to isolate RNA from a flower, seed, and leaf tissues is successful. Furthermore, this process reduces the formation of sticky pellets. The absence of reagents such as alkaline Tris buffer, β-mercaptoethanol, and a high concentration of SDS improved separation and prevented seed clumping during extraction.

To obtain pure RNA from plant tissue, the TRIzol reagent method has been modified. This reagent isolates pure, undamaged RNA that can be used immediately without the need for DNase care. We've shown that this RNA extraction approach works for maize and different grasses, as well as for all types of plant tissues. This protocol does not require specialized temperature demand but one can isolate high-quality RNA at room temperature. These findings suggest that the improved TRIzol reagent method standardized in this study is successful in extracting total RNA for gene expression transcript analysis in maize crops and other grasses species. This simple and efficient method can be used for the transcriptome analysis of plants under multiple stress conditions.

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