DOI : 10.5958/0974-0112.2015.00051.1

Indian J. Hort. 72(2), June 2015: 273-277

Short communication

RNA isolation from high polyphenol containing tissues of pomegranate

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ABSTRACT

Three methods of RNA isolation were evaluated based on quality and quantity of the RNA isolated. The methods used for RNA isolation were CTAB-LiCl method, Direct-zol™ RNA MiniPrep (ZymoResearch), and TRIzol® reagent (Life Tech.). RNA quality was assessed using spectrophotometric methods, agarose gel electrophoresis and Agilent 2100 Bioanalyzer. RNA isolation using modified CTAB-LiCl was found effective in extraction of sufficient quantity and high quality RNA from new leaves (617 µg/g FW), seedlings (636 µg/g FW) and flower buds (603 µg/g FW) having RNA integrity number (RIN) values of 8.5 each.

Key words: Pomegranate, RNA isolation, RNA intergrity number.

The currently available genomic resources for pomegranate are not sufficient to support modern breeding efforts such as marker assisted selection (MAS) or elaborate molecular studies. Extraction of high-quality total RNA is essential for the successful application of many molecular techniques, such as reverse transcription polymerase chain reaction (RT- PCR), cDNA library construction and gene expression profiling studies (Sah *et al*., 6). Several commercial reagents and kits are available for isolating RNA from plants, but they are not always effective for all plant tissues. RNA is degraded rapidly by ribonucleases (RNases) and, therefore, must be extracted quickly and efficiently (Sambrook *et al*., 8). Denaturing reagents such as phenol or guanidine thiocyanate and inhibitors of RNases such as aurintricarboxylic acid are often added to extraction buffers (Salzman *et al*., 7). Extraction of high-quality RNA from the leaves, fruits and flower buds of woody plants, like pomegranate, is particularly challenging because of high concentrations of polyphenols and other secondary metabolites found in different parts of pomegranate plant (Loulakakis *et al*., 3; Salzman *et al*., 7). RNA can form complex with phenolic compounds that render the RNA unusable for downstream applications such as reverse transcription and cDNA library construction (Salzman *et al*., 7). Thus, most of the RNA isolation methods either result in very low yields of RNA or form complexes with the contaminants resulting in low quality RNA unsuitable for first cDNA synthesis and RT-PCR. All these facts prompted us to compare RNA extraction methods using different pomegranate tissues and find out the most suitable method for pomegranate. Three isolation methods were evaluated and ranked according to the quality and quantity of the RNA obtained.

The research was carried out during 2013-14 at National Research Centre on Pomegranate, Solapur, Maharashtra, India and West Virgina State University, WV, USA. The plant tissues utilized in this work were freshly emerged pair of leaves adjacent to shoot tip (L), 15-20 day-old composite sample of whole seedling (S), 7-10 day-old flower buds (F) and peel of mature fruit of pomegranate (Fr). Samples were collected from greenhouse-grown plants and flash-freezed in liquid nitrogen, and stored at -80°C until extraction. The methods used for RNA isolation were CTAB-LiCl method (Yang *et al*., 9), Direct-zol™ RNA MiniPrep (ZymoResearch), and TRIzol® reagent (Life Tech.).

In modified CTAB-LiCl method (Yang *et al*., 9) about 200 mg of finely ground samples were transferred to 2 ml centrifuge tubes with 900 µl of extraction buffer instead [2% (w/v) CTAB, 0.1% M Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% (w/v) PVP-40] having 100 µl freshly added β-mercaptoethanol and proteinase K (80 µg/ml), not used in original protocol. The mixture was vortexed for 1 min. and then incubated for 30 min. (instead of 10 min. at room temperature in the original protocol) at 60°C, with 3-4 intermittent vortexing of tubes for 15 sec. To this mixture 800 µl chloroform was added (instead of 1 ml), shaken and centrifuged at 10,000 rpm for 10 min at 4°C. The resultant supernatant was transferred to a new tube and 800 µl of phenol:

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chloroform (1:1) was added, shaken and centrifuged for at 10,000 rpm for 10 min. (instead of 20 min. in original protocol) at 4°C. The supernatant was then transferred to a new tube, and equal volume of chloroform: isoamylalcohol (24:1) and one-third volume of LiCl (8 M) was added and kept for 4 hours at -20°C followed by centrifugation at 10000 rpm for 20 min. The pellet was successively washed with 100 and 75% ethanol (instead of 70% in original protocol). After drying for 3 min the pellet was dissolved in 250 µl of RNase free water. The RNA is treated with RNase free DNase (Life Tech.) 3 µl (10 mg/ml). Phenol: chloroform (1:1) extraction was used followed by precipitation through addition of one-tenth volume of NaOAc (3 M) and 2 volumes of 100% ethanol and incubation at -20°C for 2 h (additional step). The RNA was recovered by centrifugation at 13,000 rpm for 15 min. at 4°C followed by washing with 70% ethanol. Finally, the RNA pellet was dissolved in 40 µl RNase free water.

In Direct-zol™ RNA MiniPrep (Zymo Research) 100 mg (instead of 50 mg as per recommendation) of sample along with 1 ml TRIzol® reagent was homogenized using MagNA Lyser (Roche) at 7000 rpm, two times for 40 sec each time with flash cooling of tubes before and after first homogenization in liquid nitrogen. The mixture was centrifuged at 13,000 rpm (instead of 12,000 rpm for 1 min. as protocol) for 2 min. Liquid phase was transferred to a new microtube and centrifuged for 13,000 rpm for 10 min. (additional step). 900 µl of supernatant was transferred to Zymo-Spin™ column in a collection tube and an equal volume of absolute ethanol was added, vortexed and centrifuged for 1 min. at 13,000 rpm and it was repeated twice. About 400 hundred µl Direct-zol™ RNA pre-wash solution was added to the column and centrifuged for 1 min. at 15,000 rpm, this step was done twice. 85 µl DNase I cocktail [DNase I (5 µl), 10 X DNase I buffer (8 µl), nuclease free water (3 µl) and RNA wash buffer (64 µl)] was added and incubated at 37°C for 15 min. The mixture was centrifuged for 1 min. at 15,000 rpm (instead of 12,000 rpm). 400 µl Direct-zol™ RNA pre wash solution was added to the column and centrifuged for 1 min. at 15,000 rpm, the flow through was discarded. Thereafter, 700 µl RNA wash buffer was added to the column and centrifuged for 1 min. at 15,000 rpm (instead of 12,000 rpm) and flow through was discarded. The column was transferred to a new RNase free tube and 50 µl of RNase free water was added and centrifuged for 1 min. at 15,000 rpm to elute total RNA. Total RNA was extracted using TRIzol® reagent (Life Technologies), as per the manufacturer's instructions.

The RNA quality was assessed by three methods namely, absorbance ratio at 260/280 (Nanodrop®), agarose gel electrophoresis and Agilent 2100 Bioanalyzer RNA LabChip assay. Low absorbance ratio (less than 1.6) at 260/280 indicates the protein contamination, based on the principle that nucleic acids display an absorbance optimum at 260 nm, whereas, proteins display an absorbance optimum at 280 nm. The ratios also indirectly indicate the contamination with genomic DNA if ratios fall between 1.6-1.8. After running on an agarose gel, high quality RNA was represented by two or more sharp rRNA bands with little smearing. CTAB-LiCl and Direct-zol™ RNA MiniPrep (Zymo Research) RNA extraction methods yielded good results, RNAs from young leaves, seedlings and flower buds had RNA integrity number (RIN) value of 8.5 each, whereas yield ranged from 603-636 µg/g FW. The RIN is a related measure of RNA quality that is based on a larger portion of the electrophoretic trace (Rubio-Pina and Zapata-Perez, 5). Similarly, in Direct-zol method, both quality and quantity of RNA extracted from young seedlings and flower buds were good with RIN value of 8.6 and 8.7 and yield of 292 and 252 µg/g FW, respectively (Table 1). The ratio of 260/280 was in the range of 1.85-2.02 for young leaves, seedling and flower buds, suggesting good quality of RNA extracted using CTAB-LiCl. Similarly, the 260/280 ratio was found to be 2.02 for seedling and 1.92 for flower buds with Direct-zol™ RNA MiniPrep (Zymo Research) RNA extraction method (Table 1). However, both the methods failed to yield quality RNA from mature fruit peel. TRIzol® reagent was employed to isolate good quality RNA from high phenol containing mature fruit peel but the method could not yield desired results (Fig. 1 and 2). The 28s/18s rRNA ratio was found better in Direct-zol™ RNA MiniPrep extraction method and it ranged from 1.0 to 1.6 in different tissues (Table 1, Fig. 3). The low quality and quantity of extracted RNA may be due to high sensitivity of RNAs to various factors like contamination with protein and genomic DNA, ample presence of ribonucleases leading to RNA degradation and presence of high quantity of polysaccharides, polyphenols and other secondary metabolites. Though CTAB-LiCl method was time consuming, it yielded high quality and quantity of RNA from young leaves, seedlings and flower buds. CTAB based methods have been found effective by earlier researchers also in extraction of good quality RNA from many woody plants such as apple, peach and grapevine (Gambino *et al*., 1; Gasic *et al*., 2; Meisel *et al*., 4).

ACKNOWLEDGEMENTS

The research was funded by National Agricultural

Table 1. Comparison of yield and quality of RNA from different extraction methods using various plant parts of pomegranate.

Sample Name and No.#	Method	Agilent 2100 Bioanalyzer readings			Nanodrop spectrophotometer readings, (Thermo Scientific, USA)	
		RNA integrity No. (RIN)	RNA conc. (µg/g FW)	rRNA ratio (28s/18s)	OD value at 260/280 nm#	RNA conc. (µg/g FW)#
L1 (1)	CTAB-LiCl	8.5	617	1.1	2.00	630
S1 (2)	CTAB-LiCl	8.5	636	1.1	2.02	603
F1 (3)	CTAB-LiCl	8.5	603	1.1	1.85	449
Fr1 (4)	CTAB-LiCl	--*	81	1.1	1.50	106
L2 (5)	Direct-zol™ RNA MiniPrep	--	177	1.0	1.63	175
S2 (6)	Direct-zol™ RNA MiniPrep	8.6	292	1.6	2.02	286
F2 (7)	Direct-zol™ RNA MiniPrep	8.7	251	1.6	1.91	345
Fr2 (8)	Direct-zol™ RNA MiniPrep	--	95	1.3	1.79	179
L3 (9)	TRIzol®	--	113	1.4	1.61	176
S3 (10)	TRIzol®	--	87	0.7	1.59	149
F3 (11)	TRIzol®	--	31	0.7	1.68	49
Fr3 (12)	TRIzol®	--	26	0.0	1.16	119

*Bioanalyzer gave reading as N/A, #Av. of three observations

#L1, L2 and L3: freshly emerged pair of leaves adjacent to shoot tip; S1, S2 and S3: 15-20 day-old composite sample of seedlings; F1, F2 and F3: 7-10 days old flower buds; Fr1, Fr2 and Fr3: peel of mature fruit of pomegranate.

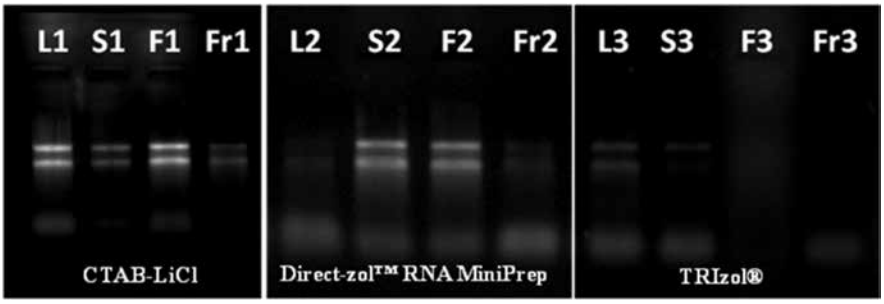

Fig. 1. Agarose gel electrophoresis of RNA extracted through different methods (L1- Fr1: RNA extracted using CTAB-LiCl method, L2-Fr2: Direct-zol™ RNA MiniPrep, L3-Fr3: TRIzol®).

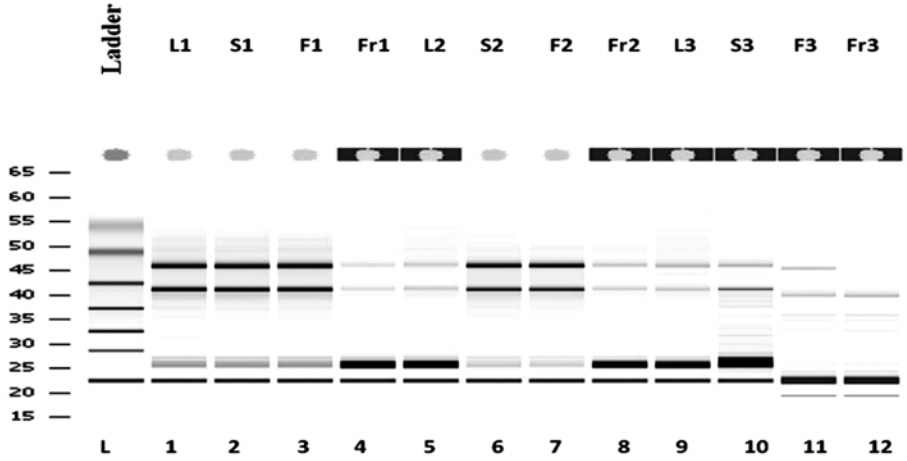

Fig. 2. Bioanalyzer gel image (L1- Fr1 and 1-4: RNA extracted using CTAB-LiCl method, L2-Fr2 and 5-8: Direct-zol™ RNA MiniPrep, L3-Fr3 and 9-12: TRIzol®).

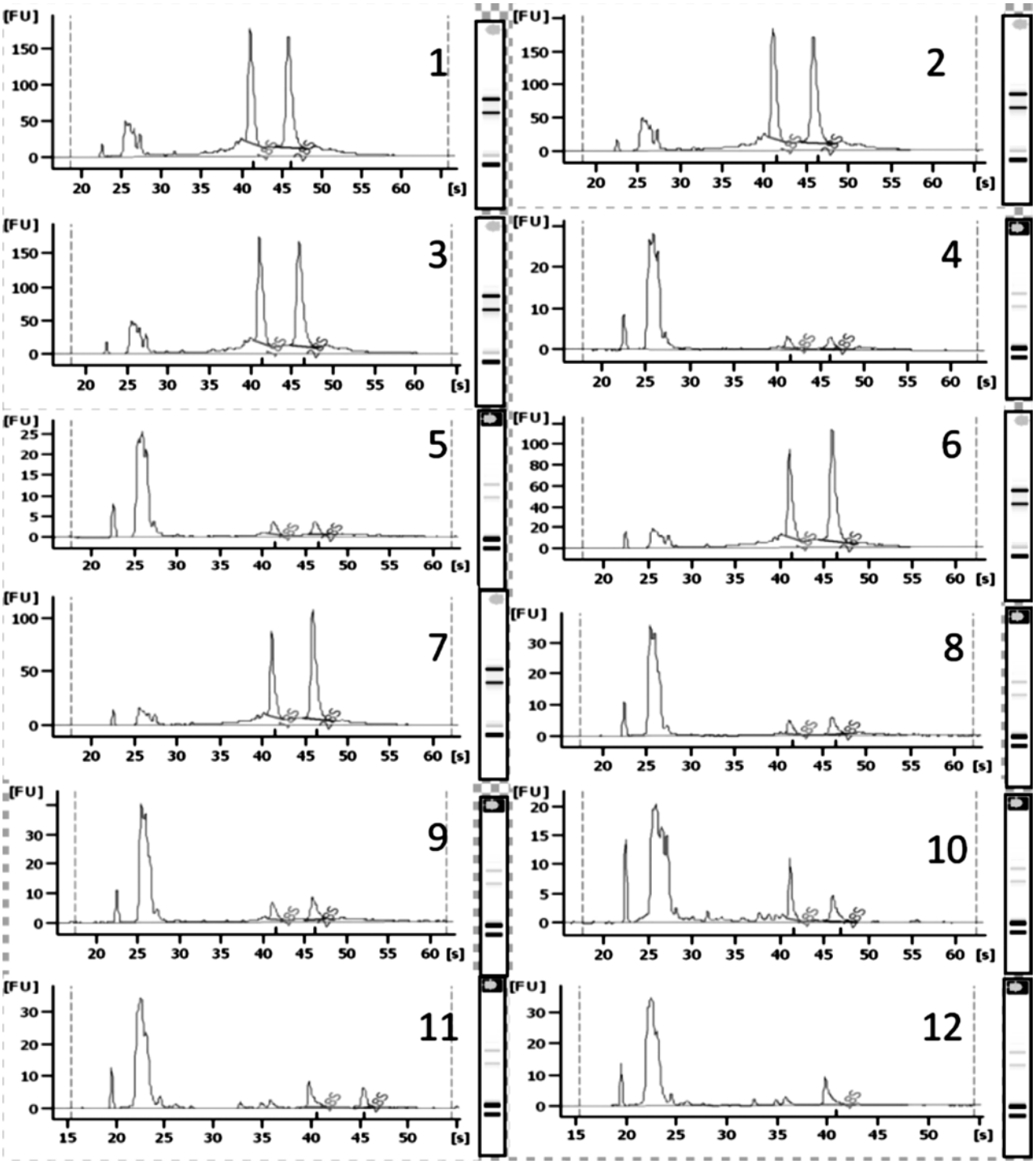

Fig. 3. Agilent 2100 Bioanalyzer electropherograms (1-4: RNA extracted using CTAB-LiCl method, 5-8: Direct-zol™ RNA MiniPrep, 9-12: TRIzol®) (note: scales differ).

Innovation Project and Indian Council of Agricultural Research. Authors are also thankful to Dr Umesh K. Reddy and West Virginia State University, USA for providing laboratory facilities.

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Received : July, 2014; Revised : March, 2015;
Accepted : May, 2015