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Efficiency of different protocols for RNA extraction from bean (*Phaseolus vulgaris* L.) leaves

Maryam Boroujerdnia[1], Mohammad Reza Bihamta[2], Khalil Alami Said[3*], Vahid Abdossi[1]

[1]*Department of Horticultural Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2]*Department of Agronomy and Plant Breeding, Faculty of Agriculture, Tehran University, Karaj, Iran*

[3]*Department of Plant Breeding, Faculty of Agriculture, Khuzestan Ramin Agriculture and Natural Resources University, Iran*

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Abstract

Purification of intact ribonucleic acids is fundamental for the study of gene expression. It has been difficult to extract a good quality and quantity of total RNA from plants with high levels of protein, phenols and polysaccharides. Therefore, Six RNA extraction method was tested to determine which of them is more efficient on bean leaves. It was observed that the CTAB-active charcoal and CTAB-SDS methods presented more pure RNA, with no degradation, suitable for further gene expression analysis. Also, quality of isolated RNA from both was further checked by cDNA synthesis. Extracted RNA was found suitable for further molecular applications such as reverse transcription and cDNA library construction.

* **Corresponding Author:** Khalil Alami Said ✉ khalilalamisaeid@mihanmail.ir

Introduction

The common bean (*Phaseolus vulgaris* L.) is the second most important commercial legume crop after soybean (Sing *et al*., 1999). This crop with near 50% of the grain legumes consumed world-wide is the most major legume for direct human diet. Beans especially in developing countries provide ~22% protein, vitamins, and minerals (Ca, Cu, Fe, Mg, Mn, and Zn) of human diets (Broughton *et al*., 2003). Therefore, *P. vulgaris* L. is currently widely planted in South, Central and North America, Africa and Asia. Bean development is adversely affected by water stress, which results in a substantial yield loss. Studying the gene expression variations in response to stress is critical for understand the molecular basis of stress adaptation in bean. Legumes generally contains high level polyphones, polysaccharides and protein that interfere with or degrade RNA, thus prevents the extraction of high quality RNA from tissues (Mattheus and Ekramoddoullah, 2003). High quality RNA extraction is first step for many molecular biology investigations methods include Northern Blot hybridization, mRNA purification, reverse transcription polymerase chain reaction (RT-PCR), cDNA synthesis, cDNA library construction and microarray analysis (Hue *et al*., 2002). Several protocols using cetyltrimethyl ammonium bromide (CTAB), Sodium dodecyl sulphate (SDS), denaturing organic solvents (phenol and chloroform), reducing agents (ß-mercaptoethanol and dithiotreitol) and Guanidinium salts (Chang *et al*., 1993; Hu *et al*., 2002; Zho *et al*., 2011; Rubio-Pina and Zapata-Peterz, 2011; Gupta *et al*., 2012; Rajakani *et al*, 2013) have been developed to isolate total RNA from tissues with high levels of polyphones and polysaccharides, but never they compare with each other.

Therefore, six methods for total RNA extraction with some modifications were compared and evaluated to develop an efficient method for RNA extraction from leaves of *P. vulgaris*.

Material and methods

Plant material

Bean plants (*Phaseolus vulgaris* L. cv. Cos 16) were grown in a growth chamber (27/24°C; 16/8h photoperiod and 60% of humidity). Leaves were harvested, immediately transferred to liquid nitrogen, and stored at -80°C until RNA extraction.

Grinding step

Samples were ground by liquid nitrogen to obtain fine powder, then transferred into sterile disposable polypropylene tubes and kept at -80 for further uses.

Solutions and Reagent

Diethyl pyrocarbonate (DEPC)–treated water (DTW) was used for all solutions, that autoclaved at 121°C for 45 min and stored at room temperature. Glasswares sterilized at +220°C for 4 hours and plastic wares autoclaved at 121°C for 45 min to inactivate RNases.

RNA isolation

CTAB-LiCl extraction method (Gasic et al., 2004)

Frozen sample powder (0.2 g) transferred to 2 ml microtube containing 1 ml CTAB extraction buffer [2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 2 M NaCl, 25 mM EDTA (pH 8.0), 2% (w/v) PVPP, 2% (w/v) β-mercaptoethanol, 0.5 g/L spermidine] mixed well and incubated at 60°C for 25 min and vortex several times. The supernatant was collected by centrifuging at 14000 rpm for 10 min at room temperature and an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, mixed well and centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was collected again and Re-extract with an equal volume of Chl:Iaa. (24:1, v/v), Centrifuge at 12000 rpm for 10 min at 4°C. The supernatant transferred to 1.5 microtube and 1/3 volume of 8 M LiCl was added, Mixed by inversion and incubated at 4°C overnight. After centrifugation at 12000 rpm for 30 min at 4°C, the RNA precipitate was washed twice with 70% ethanol and air-dry it for 5 min. RNA was dissolved in 80 µl DEPC-treated water and added 1/10 vol of 3 M sodium acetate (pH 5.5) and 2.5 vol of 100% ethanol and kept for 3 h at –80°C. After centrifugation for 20 min at 12000 rpm and 4°C, the RNA pellet was collected, washed twice with 70% (v/v) ethanol and air-dried then dissolved in 50 µl RNase-free water and stored at -80°C.

CTAB- AC method (Rajakani et al., 2013)

Transfer 0.2 g frozen sample powder to a 2 ml microtube, and add 1 ml of pre-warmed CTAB extraction buffer [2% w/v CTAB, 200 mM Tris-Cl (pH 8.0), 50 mM EDTA (pH 8.0) and 2.5 M NaCl], 0.1% activated charcoal (AC), 1.5% PVPP and 3% β-mercaptoethanol]. The ground tissue powder in buffer mixture was vortexed vigorously for 1 min, then incubated for 15 min in a water bath maintained at 65°C and centrifuged at 14000 rpm for 10 min at room temperature. Discard the supernatant, and an equal volume of Chloroform:Isoamyl alcohol (24:1, v/v) mixture was added and mixed well by inverting the tubes. Mixture was then centrifuged at 12000 rpm for 20 min at 4°C, and the upper aqueous phase was carefully collected in a fresh sterile tube. Equal volume of water-saturated Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v) was added and mixed well, followed by centrifugation at 12000 rpm for 15 min at 4°C. Upper aqueous phase was then carefully collected in a fresh tube and an equal volume of Chloroform:Isoamyl alcohol (24:1, v/v) was added, mixed and centrifuged again at 12000 rpm for 15 min at 4°C. The additional steps follow method 1 as described.

RNA extraction with spin column (Yaffe et al., 2012)

The Guanidine hydrochloride buffer [500 µl; 8 M guanidine hydrochloride, 20 mM MES hydrate and 20 mM EDTA] was added to 100 mg frozen leaf tissue in microtube and vortexed for 50 s , then centrifuged at 14000 rpm for 20 min at 4°C. The supernatant was transferred to a new microtube and add 250 µl 96% EtOH, quickly vortexed and transferred to spin column . Centrifuged the column for 1 min at 11000 rpm. Discard the flow through solution and reassemble column and collection tube. The liquid flow-through was appropriately discarded. The column was washed twice: first with 450µl 3 M Na-acetate, to remove polysaccharides, proteins and pigments, and then with 320µl 70% EtOH to remove salts. Between and after washes, the column was centrifuged at 11000 rpm and the liquid flow-through removed. The column was dried by centrifugation at maximum speed for 2 min. For elution of the RNA from the column, 40 µl of DEPC-treated water were added twice directly to the column membrane, incubated for 2 min at room temperature and centrifuged at 11000 rpm for 2 min.

CTAB- SDS method (Djami-Tchatchou et al., 2011)

Frozen sample powder (0.2 g) was transferred to 2 ml microtubes, and 1 ml of pre-warmed (65 °C) sterile extraction buffer (2% CTAB, 2% PVPP 40, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 2 M NaCl, and 400 µl 2% β-mercaptoethanol) was added, mixed well and incubated at 65 °C for 15 min to lyses the cells completely. Following the addition of 1 ml of chloroform:isoamyl alcohol (24:1 v/v) tubes were vortexed and centrifuged at 12000 rpm for 15 min at room temperature. After centrifugation, the upper phase was transferred to a new tube with an equal volume of chloroform:isoamyl alcohol, then vortexed and centrifuged again for 15 min at 12000 rpm. After the second centrifugation, the supernatants were transferred to sterile tubes following the addition of 1/3 volume of 8 M LiCl. After overnight incubation at −20 °C, the tubes were centrifuged (10000 rpm, 30 min, 4 °C) and the pellets re-suspended in 500 µl buffer 2 (1 M NaCl, 0.5% SDS, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA), pre-warmed at 60 °C and extracted with 500 µl of chloroform:isoamyl alcohol followed by a spin down at 10000 rpm for 10 min at room temperature. The upper phase (500 µl) was transferred to 2 ml microtube containing 1 ml of 100% EtOH and the RNA was precipitated at −80 °C for 1 h. After incubation for the precipitation of RNA, the tubes were centrifuged (30 min, 13 000 rpm, 4 °C). RNA pellet was recovered by washing with 1 ml of 70% EtOH (v/v) and centrifuging at 10000 rpm for 2 min, air dried and re-suspended in 80 µl DEPC water.

CTAB- ammonium acetate (Zhao et al., 2011)

Microtube containing approximately 100 mg of ground tissue and 400µl CTAB extraction buffer were homogenized in a vortex mixer and incubated for 15 min in a 65 °C water bath. After this period, the material was submitted to centrifugation at 10000g for 5 min at room temperature and the supernatant

was transferred to a new tube. Next, 400 µl of Phenol:chloroform (25:24 v/v) was added with vigorous shaking and then left to stand for 5 min at room temperature. The samples were then centrifuged for 5 min at room temperature (10000g) and the supernatant was carefully collected into a new tube. The equal volume of chloroform:isoamyl alcohol (24:1 v/v) added, then shaken vigorously and left to stand for 5 min at room temperature, and followed Centrifugation at 10000g for 5 min at room temperature. The upper phase was slowly and carefully transferred to a new tube and mixed with an equal volume of 5 M ammonium acetate (2.5M final concentration), and the mixture was then incubated on ice for at least 20 min. After centrifugation (14000g for min at 4°C), the supernatant was discarded, the pellet was washed with 70% ethanol and the solution removed by centrifugation at 14000g for 5min at 4°C. This step was repeated once to ensure that inorganic ions were removing. The pellets were dried and the RNA dissolved in 80 µl DEPC-treated water.

Guanidine hydrochloride method (Singh et al., 2011)

Ground frozen tissue (0.1 g) was transferred in to a 2 ml microtube with 500 µl extraction buffer (50 mM Tris-HCl (pH 9), 150 mM NaCl, 1% sarcosyl, 20 mM EDTA, and 5mM DTT), vortexed vigorously and then added 500µl of Phenol;chloroform;isoamylalcohol (25:24:1).The mixture was vortexed and centrifuged at 21000 g at 4°C for 5 min. Supernatant was collected and added 650 µl guanidine buffer (8 M guanidine hydrochloride, 20 mM EDTA, 20 mM MES (pH 7), and BME 200mM) and then 350 µl PCL .The contents were vortex and Centrifuged at 21000 g at 4°C for 5 min. Removed upper aqueous phase and added 500 µl of Chloroform, and centrifuge at 21000 g at 4°C for 5 min. Then, transferred to upper aqueous phase (450 µl) into 2 microcentrifuge tubes and added 45 µl of 3 M sodium acetate (pH 5.2) and 900 µl of chilled absolute ethanol ,and left at -80°C for 90 min. The RNA was collected by centrifugation (21000 g at 4°C ,20 min),followed by 70% chilled ethanol washing. The RNA pellet was dissolved in 80 µl of autoclaved DEPC water.

RNA analysis

RNA purity and concentration were assessed spectrophotometrically (NanoDrop, Technologies Inc.) by determining the absorbance of the samples at wavelengths of 230, 260 and 280 nm and by A_{260}:A_{280} and A_{260}:A_{230} ratios. RNA integrity was evaluated by electrophoresis on 1.2% (w/v) agarose gel. Ethidium bromide (EtBr) was added in the gel for band visualization under UV light by using Gel documentation.

RT-PCR

First-strand cDNA was synthesized from 1 µg of total RNA using a cDNA synthesis kit (2- steps RT-PCR Kit, vivantis) according to the manufacturer's instructions. The synthesized cDNA was used in a reaction for PCR in order to assess the quality of RNA. Reverse transcription polymerase chain reaction (RT-PCR) amplification was performed to demonstrate that the RNA could also be used for other analyses with specific primers of *fructose-bisphosphate aldolase* gene (Table 1) .The PCR program was used: 94°C for 3 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The program ended with a 10 min extension at 72°C.The amplified products were separated on a 1.2% agarose gel and visualized after staining with ethidium bromide.

Results and discussion

For a successful RNA isolation, it is necessary to prevent those polysaccharides and polyphenols which are found in different quantities in different tissues of distinct species, bind to the nucleic acids, contributing to the determination of RNA quantity, quality and integrity (Suzuki *et al.*, 2003; Zamboni *et al.*, 2008). Therefore, these parameters were evaluated to determine the most efficient RNA extraction method for bean. The A_{260}/A_{230} absorbance ratio indicates potential contamination with polysaccharides and polyphenols, while the A_{260}/A_{280} ratio indicates potential contamination with proteins (Logemann *et al.*, 1997; Manning, 1991). In many protocols, the A_{260}/A_{230} ratio higher than 2.0 indicated that the RNA was of high purity and

without polyphenol and polysaccharide contamination. The A_{260}/A_{280} ratios ranged from 1.8-2.1, indicating a lack of protein contamination.In method 1 preformed a simple and low cost RNA extraction. The total RNA yield of bean leaves was ranged from 193.3 to 212.6 ng/µl. The absorbance $A_{260}/_{230}$ ratio was lower than 2.1, suggesting that RNA samples were little contaminated by polysaccharides and polyphenols. However, the $A_{260}/_{280}$ ratio ranged from 1.86 to 2.11 (Table 2). It indicated no protein contamination. The agarose gel electrophoresis of total RNA showed two bands representing the 28S and 18S, but band of 18S was not sharp (Fig 1).

Table 1. Primers used for RT-PCR.

Gene	Accession number	Primer sequence
FW- fructose-bisphosphate aldolase	JX869951.1	5'GTCTCTCTCCCGATCCGC 3'
RV- fructose-bisphosphate aldolase	JX869951.1	5'CCTGTAGGCCTGGCGATT3'

In methods 2, activated charcoal (AC) was used for the efficient removal of polysaccharides, polyphenols and secondary metabolites. The composition of extraction buffer was including 200 mM Tris-Cl and 50 mM EDTA along with 1.5% PVPP which have strong H-receptor for binding and removal of polyphenolics (Kolosova *et al*., 2004) and 3 % β-mercaptoethanol (a higher concentration) to provide highly reducing environment to block the oxidation of polyphenolics to quinones which is known to covalently complex with nucleic acids (Lai *et al*., 2001). The cytosol-borne secondary metabolic compounds associate efficiently with activated charcoal (AC) earlier than nucleic acids, and, PVPP also has a synergistic effect in binding polyphenols on AC due to the sp2-electronic configuration of carbon rings (Bi *et al*., 1996). RNA yield and quality have been reported to be improved by increasing the ionic strength of the extraction buffer (Ghangal *et al*., 2009). Increase in the ionic strength of the extraction buffer by using high concentration of NaCl (2.5 M) in combination with LiCl precipitation effectively prevents the co-precipitation of polysaccharides along with nucleic acids. Also, the using of water saturated phenol in RNA extraction kept the pH low (acidic) which increased the RNA stability. In this protocol, yield of RNA was higher 240 ng/µl. For all samples, the $A_{260}/_{280}$ ratios ranged from 2.0-2.1, indicating a lack of protein contamination.also, the $A_{260}/_{230}$ ratio was higher than 2.0 (Table 2). This indicated that the RNA was of high purity and without polyphenol and polysaccharide contamination (Fig 1).

Table 2. Yield and purity of total RNA extracted from bean leaf with different methods.

Method	Yield of total RNA(ng/ µl)	Purity(A_{260}/A_{280})	Purity($A_{260}/_{230}$)
1	193.3-212.6	2.07-2.11	2.01-2.08
2	240-513.8	2-2.1	2.24-2.31
3	100-120	1.9-2	2-2.1
4	385-676	2.08-2.10	2.2-2.4
5	620.6-643.1	2.16-2.2	2.29-2.36
6	120-140	2	1.9

The RNA integrity was assessed by the sharpness of ribosomal RNA bands visualized on 1.2% agarose gel. For all RNA samples tested, distinct 28S, 18S bands were observed. Methods3 was a simple, economical, fast, and relatively non-toxic high-yielding method for RNA extraction. In this protocol for RNA extraction used spin column and 8 M guanidine hydrochloride buffer a salt that denatures proteins, thus inhibiting RNase, and denatures RNA .The buffer was also supplemented with 20 mM MES hydrate, to adjust the acidity for favored partitioning of RNA in the aqueous phase, and 20 mM EDTA, which served as an

RNase inhibitor. The RNA selectively binds to the silica resin in the column. Then, the RNA washed with 3 M Na-acetate to remove protein and pigment contaminants, and with ethanol to remove salts. After washing, nucleic acids are eluted from the column with water. Also, 96% ethanol (EtOH), which is known to precipitate nucleic acids (Farrell, 2004), was added 1:1 (v/v) to the samples prior to loading, to examine whether it improves RNA binding to the column.

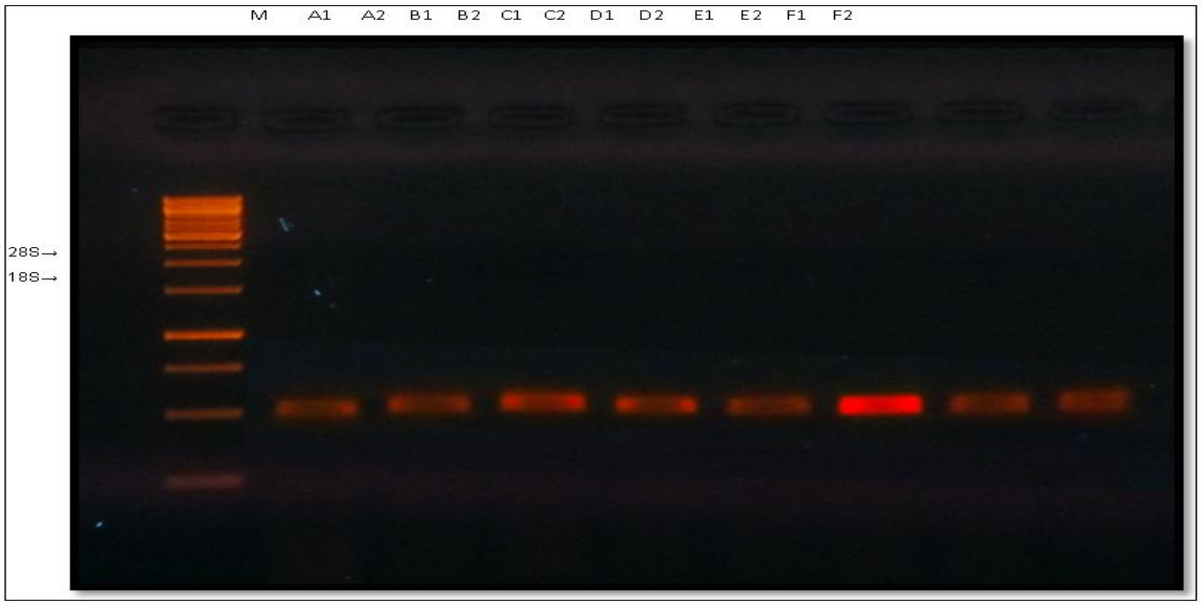

Fig. 1. 1.2% agarose gel electrophoresis of total RNA extracted from bean leaves, strained with ethidium bromide. A. Method 1; B. Method 2; C. Method 3; D. Method 4; E. Method 5; F. Method 6.

The total RNA had A_{260}/A_{280} and $A_{260}/_{230}$ ratios lower than 2.1 therefore the RNA samples were little contaminated by polysaccharides, phenol or salts but indicating a lack of protein contamination (Table 2). The yields of RNA had ranging from 100 to120 ng/µl furthermore, distinct band of 28S, 18S ribosomal RNA were observed in electrophoresis pictures (Fig 1).

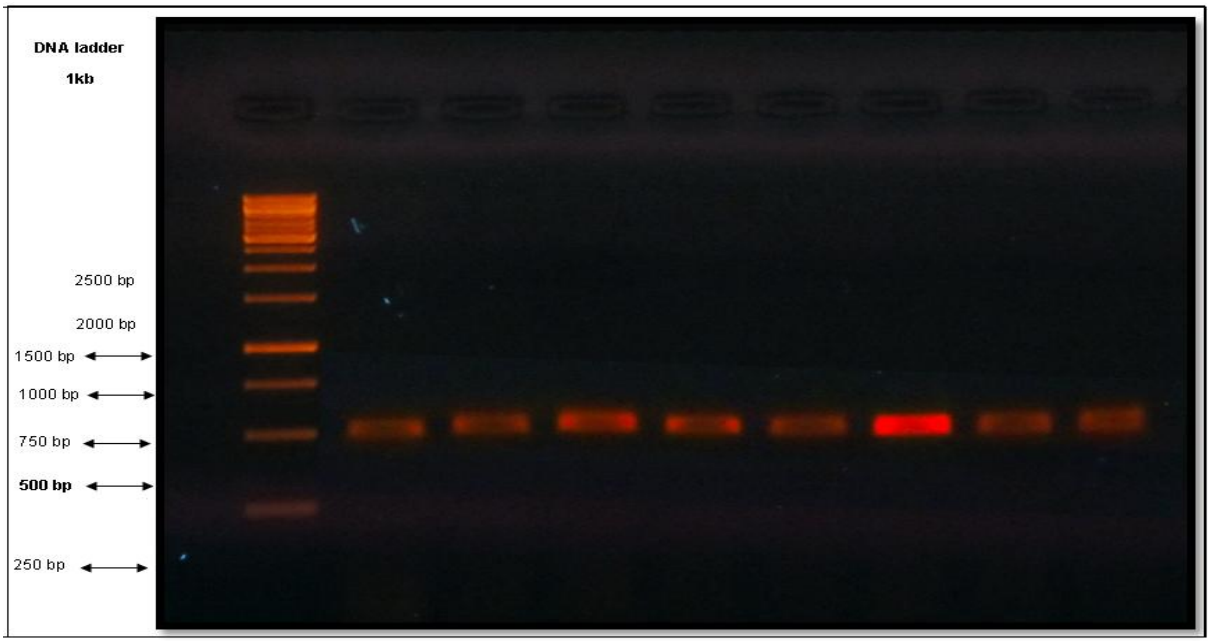

Fig. 2. RT- PCR amplification of *fructose-bisphosphate aldolase* gene using total RNA.

In protocol 4 problem of polysaccharide contamination was solved by using 2 M NaCl instead of less than 1 M in the extraction buffer and 1.0 M NaCl in the NaCl-sodium dodecylsulphate-Tris-EDTA buffer to dissolve the RNA pellet. The increase of the NaCl concentration in the buffer helps to remove polysaccharides (Fang *et al.*, 1992) and dissolves the CTAB-RNA complex, in order to allow more CTAB and polysaccharides to be removed in the chloroform extraction. Also, the precipitation step overnight at −20 °C instead of 4 °C allows high quality RNA to be recovered. The A_{260}/A_{280} absorbance ratio was 2.0 indicating that RNA was relatively free of protein contamination; and the $A_{260}/_{230}$ ratio was also higher than 2.0, indicating that RNA was of high pure and without polyphenol and polysaccharide contamination (Table 2). Furthermore, the RNA integrity was confirmed by the sharpness of ribosomal RNA bands visualized by electrophoresis which showed distinct 28S and 18S bands (Fig 1). In method 5, used ammonium acetate to precipitate RNA therefore, reducing the incubation time prior to RNA precipitation. Also, ammonium acetate selectively precipitates RNA only, leaving DNA, proteins and other nucleotides in the supernatant (Osterburg *et al.*, 1975) .It was a simple and rapid method for the isolation of total RNA compared with other methods take about a period of 2 days.This method was efficient in high yielding of total RNA (620-643.1 ng/ µl). The A_{260}:A_{280} ratios ranged from 2.16 to 2.2 in all samples, that indicating RNA samples were contaminated by proteins. The A_{260}:A_{230} ratios were from 2.29 to 2.36, indicating lack of polyphenols and polysaccharides contamination (Fig 1). The total RNA extracted showed only bands of 28 S and 5S ribosomal RNAs in 1.2% agarose gel, that weren't sharp (Table 2). In method 6 , the RNA extraction buffer was containing 150 mM NaCl and 1% sodium lauryl sarcosinate (sarcosyl), which ensured maximum RNA solubility in the aqueous phase and the removal of most polysaccharides and other insoluble material. The later addition of a guanidine hydrochloride–based buffer ensured nuclease inactivation (Logemann *et al.*, 1987; Lal *et al.*, 2001). The yield of RNA was isolated 120 to 140 ng/ ml from 100 mg of leaf tissue. An A260/A280 of 2 indicated to lack protein contamination. The A260:A230 ratio was lower of 2, indicating polyphenols and polysaccharides contamination (Table 2). The bands of 28 S and 18S ribosomal RNAs in 1.2% agarose gel weren't clearly (Fig 1).This protocol took only 3.5h thus a quick and simplified method for isolating RNA.

Conclusions

Our results showed that methods 2 and 4 were adequate for RNA extraction of bean leaves. This difference is reflected in the level of extraction yield and in that of RNA quality expressed as A_{260}/A_{280} and A_{260}/A_{230} ratios. RT-PCR was performed with specific primers of *fructose-bisphosphate aldolase* gene for the extracted RNA in methods 2 and 4. Successful amplification of *fructose-bisphosphate aldolase* from all RNA preparations indicates that the isolated RNA is of good quality and amenable for downstream applications such as cDNA synthesis and RT-PCR (Fig 2).

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