Comparative assessment of four RNA extraction methods and modification to obtain high-quality RNA from *Parthenium hysterophorus* leaf

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Abstract Isolation of high-quality RNA from weed plants such as *Parthenium hysterophorus* is a difficult task due to the hindrance caused by numerous secondary metabolites. Such metabolites not only affect the quality and yield of RNA, but also limit the quality of downstream applications. Therefore, the present study was undertaken to design a protocol for yielding RNA with better quality and quantity from *P. hysterophorus* leaf which could be suitable for functional genomics. To achieve the objective, four different important RNA extraction protocols, viz. acid guanidinium thiocyanate–phenol–chloroform, phenol–LiCl precipitation, TRIzol[®], and PVP–ethanol were tested. The PVP–ethanol method proved to be best among the tested protocols. This method was further modified for obtaining improved quality and yield of RNA. The modified method successfully enhanced the yield of RNA from 280 to 334 µg g⁻¹ fresh weight. The absorbance ratio (A_{260}/A_{280}) was in the purity range of 1.9 that indicated the good quality of RNA. To prove the feasibility of the extracted RNA in PCR-based cDNA synthesis, actin transcripts were targeted and successfully amplified using suitable primers. The improved protocol thus not only improved the yield and quality of RNA, but also gave better results in reverse transcriptase PCR.

Keywords Allelochemicals · Guanidine thiocyanate · *Parthenium hysterophorus* · RNA extraction

Introduction

Parthenium hysterophorus L. (Congress grass) is an aggressive ubiquitous annual weed. It has special morpho-physiological and biochemical adaptabilities (Ahmad et al. 2017; Patel 2011). This weed exhibits a great degree of tolerance to biotic and abiotic stresses and thus becomes an important model plant for studying the molecular mechanisms of stress tolerance and source of stress genes. However, *P. hysterophorus* synthesises numerous well known as well as novel secondary metabolites (Singh et al. 2003; Chafe 2005; Kumar. 2014) which hinders in the extraction of DNA and RNA.

As a matter of fact, abiotic stresses significantly reduce the yield of crop plants (Boyer and Westgate 2004). Drought is the most drastic among all abiotic stresses and is responsible for around 25% of the total loss to annual crop yields (Boyer 1982). Considering all the above facts, a drought-tolerant plant species could help in understanding drought-tolerance mechanisms as well as the source of anti-drought genes. *Parthenium hysterophorus* is a well-known global weed that might be used as a good source of valuable genes, proteins and metabolites. Such genes may serve as potential probes for strengthening antistress mechanism(s) or substitute for weak genes of crop plants. Although genetic engineering and transgenic approaches have opened up new ways to develop stress-tolerant plants, identification of prominent genes/transcripts that may effectively impart stress tolerance to crops is ever desirable for their possible use as ‘gene(s) of interest (*goi*)’. Such *goi* may help in developing

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transgenic crops with improved tolerance to abiotic stress. High-quality, clean and intact RNA is the key element and pre-requisite for functional genomics studies. Unfortunately, RNA molecules exhibit a high degree of susceptibility to a variety of secondary metabolites and enzymatic degradation by RNase causing problems during extraction methods (Rubio-Pina and Zapata-Perez 2011; Jordon-Thaden et al. 2015). It is of general opinion that isolation of good-quality RNA is more challenging than the extraction of DNA due to the presence of 2'-hydroxyl groups in RNA which act as nucleophiles to make RNA more susceptible to degradation (Lavoie and Abou 2008). In case of weed, RNA extraction is affected by the release of high contents of allelochemicals after disruption of the cells. *Parthenium hysterophorus* contains numerous secondary metabolites in the form of allelochemicals (Maishi et al. 1998). These inhibitory molecules might co-precipitate during RNA isolation and might interfere with downstream enzymatic reactions as observed in other species (Kolossova et al. 2004; Ma et al. 2015), varying to plant and their organ, genome and metabolome types (Wu et al. 2008). Therefore, uniform extraction methods for RNA may not be appropriate for all plants. RNA extraction protocol which is capable of yielding better quality and yield would help in making further experiments free of troubles.

In the present study, we compared the performance of four earlier reported RNA extraction methods on the leaves of *P. hysterophorus*. The most common four methods, namely of Chomczynski and Sacchi (1987), Sokolovsky et al. (1990), Salzman et al. (1999) and TRIzol® (Invitrogen) were compared in this study. The identified best method for RNA extraction, by Salzman et al. (1999), was then modified to obtain better quality RNA. The quality and functionality of the isolated RNA was confirmed by reverse transcriptase polymerase chain reaction through amplification of the actin (*ACT*) gene, responsible for cytoskeleton dynamics.

Materials and methods

Plant material and culture

The seeds of *P. hysterophorus* were sterilized with 0.1% KMnO₄ for 10 min and then washed with water ten times. The pots (6" × 6") filled with the mixture of Soilrite® (300 g/pot) were used to germinate four seeds per pot, followed by further growth for 20 days at 25 °C with a light intensity of 250 μmol photons m⁻² s⁻¹ and 85% relative humidity.

RNA extraction

Acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987)

100 mg of fresh leaves were pulverized to a fine powder using liquid nitrogen and homogenized in 1 mL buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate/pH 7.0, 0.5% (w/v) *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol. Lysate was transferred to a centrifuge tube and 0.1 mL of 2 M sodium acetate/pH 4.0 was added, mixed vigorously by inversion, then 1 mL water-saturated phenol was added and mixed by inversion, followed by the addition of chloroform/isoamyl alcohol (49:1) and mixed by shaking for 10 s. The sample was cooled on ice for 15 min and then centrifuged at 10,000×*g* for 20 min at 4 °C. The upper aqueous phase was carefully transferred to a new tube and 1 mL isopropanol was added to precipitate RNA. It was then incubated at – 20 °C for 1 h. The solution was centrifuged at 10,000×*g* for 20 min at 4 °C and the supernatant was discarded. The RNA pellet was dissolved in 0.3 mL 0.5 M TAE (Tris–acetate EDTA/pH8.0) buffer, transferred to 1.5 mL microcentrifuge tube and 0.3 mL isopropanol was added. The sample was incubated at – 20 °C for 30 min and then centrifuged at 10,000×*g* for 10 min at 4 °C. RNA pellet was dissolved with 1 mL of 75% ethanol and vortex mixed for few seconds. The sample was incubated for 10–15 min at room temperature to dissolve residual guanidinium traces, and then centrifuged at 10,000×*g* for 5 min at 4 °C. The supernatant was discarded and the RNA pellet was air dried at room temperature for 10 min. The RNA pellet was dissolved in 200 μL DEPC (0.1%)-treated distilled water. For complete solubilization, the RNA solution was incubated at 60 °C for 10–15 min. The RNA yield was determined by measuring the absorbance at 260 and 280 nm.

The phenol–LiCl precipitation method (Sokolovsky et al. 1990)

100 mg fresh leaves were pulverized to a fine powder in a mortar with liquid nitrogen. The powder was transferred to 2 mL Eppendorf tube containing 0.75 mL lysis buffer having 10 mM EDTA, 0.6 M NaCl, 100 mM Tris HCl/pH 8.0, 4% SDS and 0.75 mL Tris-saturated phenol (pH 8.0). The mixture was vortex mixed for 15–20 min and centrifuged at 10,000×*g* for 10 min. To the upper phase, 0.75 volumes of 8 M LiCl was added and stored at 4 °C overnight, followed by a brief mixing and centrifugation at 10,000×*g* for 10 min. The pellet obtained was re-suspended in 0.3 mL 0.1% DEPC-treated distilled water, mixed with 0.03 mL 3 M Na-acetate (pH 5.2) and 0.75 mL ethanol. The solution was stored at – 70 °C for 30 min,

followed by centrifugation for 10 min at $10,000\times g$. The supernatant was discarded and the pellet was washed with 70% ethanol. The RNA pellet was dried and dissolved in 200 μL DEPC-treated distilled water. The RNA purity was measured by taking the absorbance at OD 260 nm and 280 nm.

The PVP–ethanol precipitation method (Salzman et al. 1999)

0.5 g fresh leaves of *P. hysterophorus* leaves were ground to a fine powder in a mortar and pestle using liquid nitrogen. The powder was transferred to a centrifuge tube containing 5 mL buffer (0.01 g PVPP dissolved in 100 μL 2-mercaptoethanol). The solution was homogenized through vortex mixing for 1 min. An equal amount of chloroform:isoamyl alcohol (24:1) mixture was added and vigorously mixed for 10–20 min, followed by centrifugation at $16,000\times g$ for 10 min at 4 °C. The upper phase was carefully transferred to a fresh 30 mL centrifuge tube and the above steps were repeated until a homogeneous solution was obtained with no visible interphase. The supernatant was transferred to a new centrifuge tube and ethanol was added with twice the volume of homogenate and 5 M NaCl with 0.1 volume of homogenate and left overnight to precipitate at $-20\text{ }^{\circ}\text{C}$. The solution was centrifuged at $16,000\times g$ for 10 min at 4 °C and the pellet was re-suspended in 10 mL DEPC-treated water. An equal volume of Tris-saturated phenol (pH 8.0), chloroform, and isoamyl alcohol (25:24:1) was added and vortexed for 5–10 min at room temperature and centrifuged at $13,000\times g$ for 10 min at room temperature for phase separation. The upper phase was transferred to a new tube and the steps were repeated until no visible interphase accumulation of material appeared. Twice the volume of absolute ethanol and 0.1 volume of 0.5 M NaCl was added to the supernatant. The RNA was precipitated overnight. The solution containing RNA was centrifuged at $16,000\times g$ for 15 min at 4 °C and the pellet was re-suspended in 500 μL DEPC-treated water. The RNA absorbance was scanned at OD 260 and 280 nm.

The TRIzol[®] method

100 mg of the leaf sample was pulverized to a fine powder using liquid nitrogen and homogenized in 1 mL TRIzol[®] (Invitrogen, USA) reagent using a homogenizer. The lysate was centrifuged at $12,000\times g$ for 5 min at 4 °C, and then the supernatant was transferred to a new tube and incubated for 5 min to dissociate from nucleoproteins. 0.2 mL of chloroform was added to the tube and incubated for 2–3 min. The sample was centrifuged at $12,000\times g$ for 15 min at 4 °C. The upper aqueous phase containing the

RNA was transferred to a new tube. Isopropanol (0.5 mL) was added to the aqueous phase and incubated for 10 min. The sample was centrifuged at $12,000\times g$ for 10 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 1 mL of 75% ethanol. The sample was briefly vortexed and then centrifuged at $7500\times g$ for 5 min at 4 °C. The supernatant was discarded and the RNA pellet was air dried for 5–10 min, then dissolved in 40 μL of DEPC-treated water and 0.1 mM EDTA and incubated at 60 °C for 15 min. The RNA yield was determined by measuring the absorbance at 260 and 280 nm.

The solution used in the modified version of Salzman et al. (1999)

- Extraction buffer: 4.5 M guanidine thiocyanate (GITC); 125 mM Tris–HCl (pH 7.5); 30 mM sodium citrate (pH 7.4); 0.3% *N*-lauroylsarcosine; 4% glycerol; 10 mM EDTA; 3% PVPP; 4% β -mercaptoethanol (freshly added).
- Phenol:chloroform:isoamyl alcohol (25:24:1).
- 6 M LiCl solution.
- 5 M NaCl.
- Absolute and 70% ethanol.
- Diethyl pyrocarbonate (0.01%, DEPC)-treated distilled water.
- All solutions were prepared with DEPC-treated autoclaved distilled water.

RNA extraction procedure of the modified version of Salzman et al. (1999)

- Before starting the RNA isolation, the micro-pipettes, tips and tubes were thoroughly treated with DEPC-treated water and autoclaved. The mortar and pestle were dipped in 1% SDS overnight and then autoclaved.
- Half gram (0.5 g) of the fresh leaf sample was ground to a fine powder with the help of a mortar and pestle using liquid nitrogen along with PVPP (3%).
- The sample was transferred to a sterile 15 mL centrifuge tube containing 5 mL of cold extraction buffer and 200 μL of β -mercaptoethanol was added. The mixture was shaken well for 30 s and then incubated at room temperature for 5 min.
- Next, 5 mL of phenol:chloroform:isoamyl alcohol was added and mixed strongly for 15 min.
- Afterward, the mixture was spun at $15,000\times g$ for 10 min at 4 °C and the phases were separated.
- The top aqueous phase was transferred to a 15 mL sterile centrifuge tube and an equal volume of phenol:chloroform:isoamyl alcohol was added.

- The sample was shaken for 30 s and spun at $15,000\times g$ for 10 min at 4 °C.
- The clean supernatant was transferred to 15 mL sterile centrifuge tube containing double volume of absolute alcohol and half volume of NaCl (5 M).
- The above whole preparation was incubated at – 20 °C for at least 6 h.
- The mixture was spun at $15,000\times g$ for 15 min at 4 °C.
- The pellet was obtained and re-suspended in 5 mL of DEPC-treated autoclaved water.
- The resulting mixture was shaken thoroughly with an equal volume of phenol:chloroform:isoamyl alcohol for 10 min at room temperature.
- Later, the mixture was spun at $10,000\times g$ for 10 min at room temperature.
- Two phases were formed; the upper phase was carefully transferred to a sterile centrifuge tube containing double the volume of absolute ethanol and half the volume of LiCl (6 M) and then the mixture was incubated at – 20 °C for at least 6 h.
- The chilled mixture was centrifuged at $15,000\times g$ at 4 °C for 10 min.
- The pellet was obtained and washed with 70% ethanol and then air dried at room temperature, although complete drying of the RNA pellet was avoided which otherwise makes RNA re-suspension difficult.
- Finally, the pellet was re-suspended in 100 µL DEPC-treated autoclaved distilled water.

Quantification of total RNA

The RNA containing the aliquot (5 µL) was added to 495 µL DEPC-treated autoclaved distilled water and vortex mixed. The absorbance was read at 260 and 280 nm using a spectrophotometer (SICAN 2301, India). DEPC-treated autoclaved distilled water was used as a blank. The ratio of $OD_{260\text{ nm}}/OD_{280\text{ nm}}$ and $OD_{260\text{ nm}}/OD_{230\text{ nm}}$ provides an estimate of the purity of nucleic acid. Pure preparations of RNA have an OD_{260}/OD_{280} ratio of 1.8–2.0 (Sambrook et al. 1989).

The RNA concentration (µg/µL) was calculated using the following formula:

$$\text{RNA concentration (}\mu\text{g}/\mu\text{L)} = \frac{OD_{260} \times \text{dilution factor} \times 40}{1000}.$$

Qualitative analysis of total RNA

Qualitative analysis of total RNA isolated from fresh leaves was done by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide ($0.5\text{ }\mu\text{g mL}^{-1}$) and documented with the help of a gel documentation system (Bio-Rad, USA).

Reverse transcriptase polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized from 2.5 µg of total RNA using other reactants as follows.

Total RNA (2.5 µg in 1.5 µL), oligo dT primer (18 mer, 0.5 µg in 1 µL) and DEPC-treated water (0.1%, 10 µL) were mixed gently, spun briefly and incubated at 65 °C/ 5 min. It was followed by chilling on ice for 1 min, briefly spun and placed on ice. A mixture containing 4 µL reaction buffer, Ribolock RNase inhibitor (20 units in 0.5 µL), dNTPs mix (2 µL of 10 mM) and M-MuLV reverse transcriptase (200 units in 1 µL) was prepared, gently mixed and briefly spun. This reaction mixture was incubated at 42 °C for 60 min. The reaction was terminated by heating at 70 °C/10 min. Finally, the first strand of cDNA was synthesized and stored at – 20 °C till further use in PCR.

PCR amplification

To assess the quality of RNA, RT-PCR was performed with primers of actin gene. The PCR conditions consisted of initial PCR activation and denaturation for 5 min at 94 °C, followed by 35 cycles of (i) denaturation at 94 °C for 1 min, (ii) annealing at 52 °C for 1 min and (iii) extension at 72 °C for 1 min. The final extension step was performed at 72 °C for 10 min. The PCR product picked with actin gene-specific primers (left: 5'ACCTTACCGA C TCACTGATG3' and right: 5'AGATCCACATCTGT TGGAAG3' both with T_m 55° C) was run and analyzed on 1.2% (w/v) agarose gel and visualized using ethidium bromide ($0.5\text{ }\mu\text{g mL}^{-1}$) dye. A total of 12 µL (containing 2 µL DNA loading dye + 10 µL of PCR product) of the mixture was loaded in wells; 100 bp DNA ladder was used as a marker.

Results and discussion

The present work demonstrates the comparative analysis of four RNA extraction protocols employed on *P. hysterophorus* leaf. The selected four RNA extraction methods were different from each other. The protocol of Chomczynski and Sacchi (1987) and TRIzol® (Invitrogen, USA) were commonly used for all types of tissues. The protocol of Salzman et al. (1999) is commonly used for plant tissues, whereas Sokolovsky et al. (1990) is in common use for fungi. A number of modifications in original procedures have been reported for isolation of RNA from diverse plant systems (Accerbi et al. 2009). *Parthenium hysterophorus* could not yield a high grade of RNA (Fig. 1) in satisfactory quantities by the existing methods, perhaps due to the presence of high content of terpenoids, phenolics and

Fig. 1 Total RNA extracted from *P. hysterophorus* leaf using different RNA isolation methods and 3 μg of total RNA from each method were loaded on 1% agarose gel stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$)

polysaccharides. Several plants have been reported to have allelopathy character with a specific composition of secondary metabolites that hampers the re-suspension of precipitated RNA and also affects its storage, quality as well as quantity (Ding et al. 2008). RNA isolated from *P. hysterophorus* leaf by existing methods was partially degraded and was difficult to dissolve (Fig. 1). Oxidation of homogenate might contribute to this poor quality of RNA. Oxidation of phenolic compounds and co-precipitation of polysaccharides are supposed to interact irreversibly with the RNA molecule (Chan et al. 2004; Yockteng et al. 2013).

The first method by Chomczynski and Sacchi (1987) was used in this study which is generally used for RNA extraction from leaf tissues. However, in case of *P. hysterophorus*, it failed to give satisfactory results perhaps due to the weak precipitation and shearing of RNA. The RNA extraction method of Sokolovsky et al. (1990) was used as a second method in the present study. This method did not produce quality RNA from *P. hysterophorus* leaf. Separation of the 28S and 18S bands was not clear as confirmed by gel electrophoresis. Next, TRIzol® (Invitrogen, USA)-based RNA extraction was carried out, but RNA yield and integrity still remained unsatisfactory. Such grades of RNA do not produce satisfactory results in high-throughput molecular biology research. RNA isolation protocol of Salzman et al. (1999) was modified by adding an appropriate amount of EDTA, glycerol, and changing

Table 1 Comparison of the ingredients of the two RNA extraction methods including the method of Salzman et al. (1999) and the one developed through its modification in the present study

Salzman et al. (1999)	Modified protocol
4.0 M Guanidine thiocyanate	4.5 M Guanidine thiocyanate
100 mM Tris-HCl (pH 8.0)	125 mM Tris HCl (pH 7.5)
25 mM Sodium citrate (pH 8.0)	30 mM Sodium Citrate (pH 7.4)
0.5% <i>N</i> -lauroylsarcosine	0.3% <i>N</i> -lauroylsarcosine
2% β -mercaptoethanol	4% β -mercaptoethanol
Nil	4% Glycerol
Nil	10 mM, EDTA
PVP (1%)	PVPP (3%)
Tissue:buffer (w/v) ratio = 1:5	Tissue:buffer (w/v) ratio = 1:10

concentration of some components (Table 1) as well as duration of treatment. These modifications enhanced the integrity and purity of the RNA extracted from *P. hysterophorus* leaf.

Polyvinylpyrrolidone (PVPP) is a highly cross-linked modification of polyvinylpyrrolidone (PVP) and hence more effective in eliminating phenolics, terpenoids and polysaccharides (released during cell lysis) through adsorption (Sajeevan et al. 2014). The duration of the precipitation period with absolute alcohol at low temperature was increased resulting in a good yield of RNA molecules. Glycerol was used in the modified protocol for eliminating lipid contaminations from the extract that further improved the purity of nucleic acids (Shojaie et al. 2014). The procedure includes lysis and homogenization of sample in the presence of EDTA-containing buffer, which immediately inactivates RNases to ensure isolation of the intact RNA. An almost white and water-soluble RNA precipitate was obtained by the modified protocol from *P. hysterophorus* leaf. The clear and distinct bands of 28S and 18S rRNA in agarose gel, containing ethidium bromide ($0.5 \mu\text{g mL}^{-1}$), indicated the effectiveness of the modified protocol (Fig. 1). Quality as well as quantity of the extracted RNA was also examined spectrophotometrically and the values of A_{260}/A_{280} and A_{260}/A_{230} ratio obtained were 1.99 and 2.09, respectively (Table 2), suggesting the presence of very low concentration of interfering substances. Variations in the extraction buffer composition and other conditions are supposed to ultimately influence the PCR product quality and downstream process of molecular biology.

The total RNA used as a template in the present amplification reaction to synthesize the cDNA strand was of good quality and free from DNA contamination. Sufficient quantity of cDNA was produced by reverse transcription and further confirmed by good amplification of the target sequence. In the present study, amplification of

Table 2 Quantification of total RNA extracted by different methods from the leaves of *Parthenium hysterophorus*

Protocol used	Concentration of RNA ($\mu\text{g}/\mu\text{L}$)	Concentration of RNA ($\mu\text{g}/\text{g}$ FW)	A_{260}/A_{280}	A_{260}/A_{230}
Chomczynski and Sacchi (1987)	1.44 ± 0.07	288 ± 21	1.71 ± 0.009	1.78 ± 0.018
Sokolovsky et al. (1990)	1.20 ± 0.06	248 ± 17	1.63 ± 0.007	1.72 ± 0.014
Salzman et al. (1999)	1.40 ± 0.18	280 ± 19	1.75 ± 0.012	1.81 ± 0.018
TRIzol® (Invitrogen)	1.34 ± 0.04	268 ± 16	1.68 ± 0.010	1.74 ± 0.012
Modified protocol	1.67 ± 0.09	334 ± 24	1.99 ± 0.014	2.09 ± 0.021

$n = 3$, $P \leq 0.05$

FW fresh weight

Fig. 2 Reverse transcriptase polymerase chain reaction (RT-PCR) amplification of the actin gene on 1% agarose gel stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) and arrows indicate the actin gene product; DNA ladder (100 bp)

520 bp housekeeping actin gene was performed (Fig. 2) using the *P. hysterophorus* cDNA.

In conclusion, the present modified RNA extraction protocol proved to be efficient for the extraction of improved quality of RNA from the leaf of *P. hysterophorus*. Sharp intensity and clear resolution of RNA bands on agarose gel and absorbance ratio ($A_{260}/A_{280} = 1.9$ for RNA) indicated a clear merit over existing protocols. This protocol can be used successfully for RNA isolation from

the leaf tissue containing high amount of allelochemicals, such as tissue of a weed *P. hysterophorus*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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