**Extraction of functional total RNA from *Cayratia trifolia***

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**ABSTRACT**

RNA extraction is the initial and can be the most critical step in the study of gene expression. Extraction of RNA from *Cayratia trifolia* plant tissue which contains high levels of polysaccharides, polyphenols and other secondary metabolites is tedious and difficult. We had tested a commercial RNA extraction kit, RNeasy Plant Mini Kit (Qiagen) and made some modification. The use of both RLT and RLC buffers did not produce any usable RNA. Addition of PEG to the buffers improved RNA yield. However, RLC buffer with PEG 8000 gave better yield compared to RLT buffer with PEG 8000. RNA analysis on agarose gel indicated the presence of 2 clear and distinct 18S and 25S rRNA bands proven that the RNA samples are of high quality. Spectrophotometry analysis on RNA samples showed the OD260/230 and OD260/280 ratios were more than 1.8. cDNA display experiments indicated the presence of differentially displayed bands. These results proved that the RNA obtained were functional and of a good quality.

**Key words:** RT-PCR, differential display, PEG 8000, cDNA, rRNA

**Introduction**

RNA isolation is the initial step involved in the study of gene expression and also in the utilization of genes for genetic improvement. Any RNA isolation protocol must succeed in extracting RNA species from cellular and extra-cellular components. Therefore, it is crucial to eliminate DNA, protein, polysaccharides, phenolic compounds and other secondary metabolite compounds during the RNA extraction process. Inability to obtain clean, intact RNA may result in the failure of downstream applications such as cDNA synthesis, RT-PCR, cDNA display and cDNA synthesis.

The extraction of RNA from plant tissues can be complicated and most of the time requires modification of existing protocols or development of novel procedures. In this study, we used *Cayratia trifolia* as a plant material. *C. trifolia* plant was selected based on preliminary data gathered by our group which showed that the plant is capable of accumulating high amount of heavy metals and hydrocarbon. Our long term research objective is to isolate genes which confer plant resistance to heavy metals and hydrocarbons. *C. trifolia* is well known for their diversity in secondary metabolite, polysaccharide, and polyphenolic compounds [1,15]. All of these components would co-precipitate with the RNA and constitute the major obstacle of RNA isolation and also often hinder RNA preparation or compromise RNA sample quality [6,10]. Many of the established RNA isolation protocols are based on highly toxic chaotropic agents such as phenol, phenol/chloroform or guanidine thiocyanate which quickly denature the endogenous ribonucleases in tissues [8]. However, these methods often failed to significantly eliminate carbohydrate and phenolic compounds resulting in a poor RNA yield.

We developed a method to isolate good quality RNA from leaves of *C. trifolia* after significant modification of the method from RNeasy Plant Mini Kit established by Qiagen. High molecular weight polyethylene glycol (PEG 8000) was added in the extraction buffer (RLC buffer) to bind to the phenolic compounds which were then eliminated by ethanol precipitation [12]. We proved that the isolated RNA is of high quality and quantity by optical density and agarose gel analyses. Results from RT-PCR experiment confirmed that RNA was functional.

**Materials and methods**

**Plant material:**

*C. trifolia* plants were collected from Petronas Penapisan Melaka Sdn. Bhd. (PPMSB), Melaka, Malaysia. The plants were grown in the Universiti Kebangsaan Malaysia’s greenhouse under normal soil and petroleum sludge. Fresh leaves were
collected and labeled according to the habitat (sludge and normal soil). Leaves were then cleaned with sterile distilled water and used for RNA extraction.

**Isolation of RNA:**

The protocol was as described by the RNeasy Plant Mini Kit (Qiagen) instruction manual. All the equipments and solutions used in the protocol were treated with diethylpyrocarbonate (DEPC) as described by Sambrook et al. [16]. PEG 8000 was added to the extraction buffer (RLC buffer) to a final concentration of 2% β-mercaptoethanol was added into RLT and RLC buffers with the ratio of 10 μL β-ME per 1 mL buffer and properly mixed. Four volumes of 100% undenatured ethanol were added into RPE buffer.

**Determination of RNA quality and quantity:**

The quality and quantity of RNA were analyzed by measuring optical density (OD) using Biophotometer (Eppendorf AG, Germany). RNA quantity was determined by diluting 1 μl of RNA sample into 49 μl of sterile DEPC-treated water and OD was measured at 260nm wavelength. RNA quality was determined by measuring OD at 230 nm, 260 nm and 280 nm. The rationings of OD_{260}/OD_{230} and OD_{260}/OD_{280} were then calculated. The quantity and quality of RNA were also determined by electrophoresis on 1.2 % (w/v) agarose gel. The gel was stained with ethidium bromide [16] and visualized using Vilber Lourmat Infinity-Cap gel imager (Germany).

**Reverse transcription:**

A good quality total RNA was used for the first strand cDNA synthesis using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to the manufacturer recommendations. Oligo(dT) primer was used as a primer for cDNA synthesis by SuperScript III Reverse Transcriptase. A total of 3μg RNA was used as a template.

**Second strand cDNA synthesis and differential cDNA display:**

Differential cDNA display was performed using GeneFishing™ DEG Premix Kit (Seegene) according to the instruction manual provided. Fifty ng of the first-strand cDNA was used as a template for the second strand cDNA synthesis. The reaction mixture consisted of 5 μM of one of the 20 arbitrary ACP primers (ACP1-ACP20) (Seegene), 10 μM dT-ACP2 and SeeAmp™ ACP™ Master Mix. The mixture was incubated at 94°C for 5 minutes and 50°C for 3 minutes for the second strand cDNA synthesis reaction to take place. Final incubation at 72°C for 1 minute was required for the reaction to complete. The mixture was then subjected to PCR for 40 cycles at 94°C for 40 second, 65°C for 40 second and 72°C for 40 second followed by final incubation at 72°C for 5 minutes. The PCR products were separated on 2% (w/v) agarose gel and stained with ethidium bromide as recommended [16].

**Results and discussion**

A commercially available RNeasy Plant Mini Kit for RNA extraction was tested for its efficiency to extract total RNA from the leaves of *C. trifolia*. The kit was supplied with two lyses buffer, i.e. RLC and RLT. Both buffers were tested in addition to a slight but significant modification by addition on PEG 8000 to a final concentration of 2%. Figure 1 shows the total RNA samples obtained from the procedure. RNA extraction from leaves of *C. trifolia* grown in normal soil and sludge using unmodified RLT buffer produced a very poor and unusable RNA (CT1 and CT2). Addition of PEG 8000 to RLT buffer slightly improved yield of RNA from both *C. trifolia* leaves (CTP1 and CTP2).

Change of lyses buffer from RLT to the RLC buffer did not produce any RNA product (CC1 and CC2). However, addition of PEG 8000 to RLC buffer produced a significantly very low RNA extraction using unmodified RLT buffer showed the presence of 2 clear and distinct bands proved that the RNeasy Plant Mini Kit was a very efficient system for RNA extraction.

A summary of spectrophotometry analysis of RNA samples is shown in Table 1. RNA samples obtained from tobacco and *C. trifolia* leaves extracted with the presence of PEG 8000 were of a good quality as indicated by the ratio for both OD_{260/280} and OD_{260/230} of more than 1.8. RNA extractions from *C. trifolia* leaves without the use of PEG 8000 produced a significantly very low RNA concentration which was not detected on agarose gel (Figure 1).

The RNeasy Plant Mini Kit provides a choice of lyses buffers, i.e. RLT and RLC, which contain guanidine thiocyanate and guanidine hydrochloride respectively. In most cases, RLT buffer is the lyses buffer of choice due to the greater cell disruption characteristic and denaturation properties of guanidine thiocyanate. Many established works used

Fig. 1: Agarose gel electrophoresis of total RNA from *C. trifolia*. TB, tobacco RNA with RLT buffer; CT1, *C. trifolia* (normal soil) RNA with RLT buffer; CT2, *C. trifolia* (petroleum sludge) RNA with RLT buffer; CTP1, *C. trifolia* (normal soil) RNA with RLT buffer and PEG; CTP2, *C. trifolia* (petroleum sludge) RNA with RLT buffer and PEG; CC1, *C. trifolia* (normal soil) RNA with RLC buffer; CC2, *C. trifolia* (petroleum sludge) RNA with RLC buffer and PEG; CCP1, *C. trifolia* (normal soil) RNA with RLC buffer and PEG; CCP2, *C. trifolia* (petroleum sludge) RNA with RLC buffer and PEG. 5 µl of each RNA products were loaded in each well.

RLT for RNA extraction from leaves and gave good quality of RNA [9,13,14]. However, due to the amount and the type of secondary metabolites presence in *C. trifolia* tissue [1,15], the use of guanidine thiocyanate is not recommended as it can cause solidification of the sample, making extraction of RNA impossible.

Different protocols often produce different quality of RNA preparation. Towë *et al.* [18] found that a better preparation of RNA was obtain by applying phenol:chloroform during extraction procedure. Dineen *et al.* [3] had tested 5 different types of commercial kits to extract RNA from virus and all of them produced different qualities of RNA preparation. Djami-Tchatchou and Straker, [4] used cetyltrimethylammonium bromide-based protocol without using phenol to extract RNA from skin and flesh of avocado fruit. Gehrig *et al.* [5] found that the use of PEG 8000 during RNA extraction greatly improved the yield and served as a better template for reverse transcription and PCR amplification. PEG 8000 is a reducing agent that eliminates the polysaccharides and prevents the oxidation of phenolic compounds. Phenolic and other compounds that may interfere with RNA were bound to PEG 8000 in extraction buffer and subsequently eliminated by precipitation as an initial step to RNA extraction [2,7]. In cells, total RNA consists mainly of mRNA, tRNA and rRNA which constitute approximately 3%, 17% and 80% respectively. During extraction, the ratio of RNA recovery is also followed by the ratio of different types of RNA in cells. Therefore, a good quality of RNA preparation is often indicated by the presence of clear 25S and 18S rRNA bands. Our results (Figure 1) again proved that the RNA recovery and yield were greatly improved by the addition of PEG 8000 into the lyses buffer.

RNA obtained from these modified method served as a robust template for reverse transcription as indicated by PCR amplification. Figure 2 shows results of cDNA display from *C. trifolia* grown in sludge compared to *C. trifolia* grown in normal soil. PCR products analysed on 2% (w/v) agarose gel showed the presence of differentially display cDNA bands. Twenty cDNA bands were either qualitatively or quantitatively displayed in *C. trifolia* grown in sludge. Meanwhile 7 cDNA bands were either qualitatively or quantitatively displayed in *C. trifolia* grown in normal soil.

The presence of differently display cDNA bands indicated that genes representing the particular cDNA were differently expressed in that particular sample compared to others. Petroleum sludge contains high concentration of toxic components such as heavy metals [11] and hydrocarbons [17]. In our case, differently expressed genes in *C. trifolia* grown in sludge may represent genes involved in plant resistance mechanism or genes expressed due to plant stress against toxic compounds of either heavy metals or hydrocarbons that presence in the sludge. These results proved that the total RNA obtained from the extraction protocol was of high quality. In the future, we will clone and identify these cDNAs. These cDNAs may represent genes of interest particularly the one that could be used in bioremediation.
Fig. 2: Differentially displayed cDNA fragments of *C. trifolia* grown in normal soil (a) and petroleum sludge (b). Sample 1-5 were generated with combinations of one of ACP1-ACP5 primer and dT-ACP2 primer respectively. Sample 2-10 were generated with combinations of one of ACP6-ACP10 primer and dT-ACP2 primer respectively. Sample 11-15 were generated with combinations of one of ACP11-ACP15 primer and dT-ACP2 primer respectively. Sample 16-20 were generated with combinations of one of ACP16-ACP20 primer and dT-ACP2 primer respectively. M, 100 bp DNA ladder (Vivantis). Circles indicate differentially displayed cDNA. *C. trifolia* grown in normal soil is indicated by a. *C. trifolia* grown in petroleum sludge is indicated by b.

Table 1: RNA quality measured by UV spectrophotometry.

<table>
<thead>
<tr>
<th>Leaf samples</th>
<th>Type of lysis buffer used</th>
<th>OD_{260/280}</th>
<th>OD_{260/230}</th>
<th>RNA conc. (μg/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco - TB</td>
<td>RLT</td>
<td>2.00</td>
<td>1.90</td>
<td>109.15</td>
</tr>
<tr>
<td><em>C. trifolia</em> (normal soil) – CT1</td>
<td>RLT</td>
<td>1.54</td>
<td>0.469</td>
<td>3.04</td>
</tr>
<tr>
<td><em>C. trifolia</em> (sludge) – CT2</td>
<td>RLT + PEG 8000</td>
<td>1.98</td>
<td>1.85</td>
<td>37.87</td>
</tr>
<tr>
<td><em>C. trifolia</em> (normal soil) – CTP1</td>
<td>RLT + PEG 8000</td>
<td>1.80</td>
<td>1.82</td>
<td>44.18</td>
</tr>
<tr>
<td><em>C. trifolia</em> (sludge) – CTP2</td>
<td>RLC</td>
<td>1.61</td>
<td>0.50</td>
<td>4.00</td>
</tr>
<tr>
<td><em>C. trifolia</em> (sludge) – CC1</td>
<td>RLC + PEG 8000</td>
<td>1.58</td>
<td>0.64</td>
<td>5.60</td>
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<tr>
<td><em>C. trifolia</em> (normal soil) – CCP1</td>
<td>RLC + PEG 8000</td>
<td>1.94</td>
<td>1.85</td>
<td>64.80</td>
</tr>
<tr>
<td><em>C. trifolia</em> (sludge) – CCP2</td>
<td>RLC + PEG 8000</td>
<td>1.98</td>
<td>1.90</td>
<td>86.84</td>
</tr>
</tbody>
</table>
Conclusion:

We have successfully isolated total RNA of a good quality from *C. trifolia* by modification of method established by Qiagen. cDNA display experiments showed the presence of display bands probed that the RNA was functional.

Acknowledgement

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References